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Title:
Studies on Escherichia coli O157:H7 in sheep

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in

Sheep

Andrew Derek Wales

A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy (Ph.D.) in the Faculty of Medicine, Department of Pathology and Microbiology, in December 2002.

Seventy-one thousand words.

Abstract

Escherichia coli O157:H7 is a significant human pathogen that persists in asymptomatic animal hosts. It forms a characteristic attachment, the attaching-effacing (AE) lesion, on cell monolayers *in vitro* and in the intestine in some animal models. Cattle and sheep are asymptomatic carriers of *E. coli* O157:H7 and sources of the organism for humans. The present studies examined the persistence of several strains of *E. coli* O157:H7 in orally inoculated sheep, and attempted to correlate persistence with features of the strains *in vitro* and *in vivo*. A particular hypothesis tested was that adhesion of the bacterium to the intestinal mucosa is a significant mechanism for persistence in animal hosts.

Host- and strain-dependent variation in the persistent excretion of *E. coli* O157:H7 was observed. Correlations could not be discerned between the persistence of the various bacterial strains and the results of a range of phenotypic tests. The ability of *E. coli* O157:H7 to form AE attachments to the large intestinal mucosa in sheep of up to six months of age was demonstrated. No consistent site of persistence of *E. coli* O157:H7 within the ovine alimentary tract was found, and AE lesions were not detected in sheep which were persistently excreting the organism. However, commensal bacteria, including *E. coli* O26, were seen to have formed AE attachments on the large intestinal mucosa.

It was concluded that the attachment of *E. coli* O157:H7 to the large intestinal mucosa by AE lesion formation may have a role in persistent carriage, but that persistence of the bacterium in the ovine intestine is probably influenced by additional bacterial and host factors. The potential value of interference in the formation of AE lesions, to reduce the prevalence of *E. coli* O157:H7 excretion by sheep and other ruminants, merits further investigation.

Dedication and acknowledgements

The Author gratefully acknowledges the opportunity to perform the present studies and the encouragement and guidance throughout, provided by my supervisors Dr. Geoffrey R. Pearson and Professor Martin J. Woodward. The support and encouragement of family and friends, particularly my wife Catherine, has been invaluable.

The work described was performed in collaboration with the Aerobiology unit, University of Bristol, Langford and the Department of Bacterial Diseases, Veterinary Laboratories Agency (VLA), New Haw, Addlestone, Surrey. The PhD project was funded by the VLA Seedcorn funding programme, project SC0081, within the Department for the Environment, Food and Rural Affairs (DEFRA, formerly MAFF)-funded Food Safety and Zoonoses programme, project OZ0706.

Primary collaborators were: Mrs Christine M. Hayes and Dr John M. Roe (Aerobiology); Dr Geoffrey R. Pearson (Comparative Pathology, University of Bristol, Langford); Dr Adrian L. Cookson, Dr Felicity A. Clifton-Hadley, Dr Michael P. Dibb-Fuller and Professor Martin J. Woodward (VLA). The expert technical assistance of the following is gratefully acknowledged: Mr Derek Clifford (animal services; VLA), Mr John Conibear (photography; Langford), Mr Bill Cooley (scanning electron microscopy; VLA), Mr Keith Hiley (animal services; VLA), Mrs Sheila Jones (immunoperoxidase staining; Langford), Mr David Sheehan (animal services; VLA), Mr Andy Skuse (transmission electron microscopy, information technology; Langford) and Mrs Ginny Thomas (histopathology; Langford).

Specific contributions are summarised below.

Pathology

Mrs Ginny Thomas assisted with cutting and H&E staining of sections. Special stains were done by staff in the Comparative Pathology Laboratory, Langford. A. Skuse provided expertise in the retrieval and processing of tissues for electron microscopy, and in the operation of the transmission electron microscope.

Bacteriology

A. Cookson and C. Hayes performed most of the IMS processing and culture. C. Hayes additionally devised and trialled the non-IMS culture methods detailed in Section 6.2.2. Genotyping of the O115:H- strain (Section 6.3.2.2) was done by A. Cookson. Genotyping of O157 organisms recovered in Experiment 6/4 was done by R. LaRagione and A. Best.

In vitro studies

M. Dibb-Fuller assisted with PCR examinations, and with quantiative adhesion, FAS and inorganic acid tolerance studies. M. Dibb-Fuller also developed the bovine cell cultures. A. Cookson assisted with generation of antibiotic-resistant strains, Western blotting and FAS studies. A. Cookson performed all growth in mixed culture studies (Section 3.5.4). All cell monolayers examined by SEM were processed, examined and photographed by the VLA EM unit. R. LaRagione assisted with cell monolayer studies.

In vivo studies

The housing, care and faeces sampling of animals and the operation of animal facilities were performed by the Animal Services Unit (VLA) and by J. Roe and C. Hayes. A. Cookson and C. Hayes prepared inocula and assisted with tissue sampling and processing. F. Clifton-Hadley, M. Dibb-Fuller, R. LaRagione and Ms Katherine Sprigings assisted with post-mortem and terminal anaesthestic sampling procedures. Animals were anaesthetised by Dr Kate White (ligated loop studies) and J. Roe. Mr Steven J. Spencer performed the ligated loops surgery.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original, except where indicated in the acknowledgements and by special reference in the text, and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED: 

DATE: 11th December 2002

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List of abbreviations used

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AE	Attaching-effacing	1.1.3
AEC	Amino ethylcarbazole	2.5.4.3B
AEEC	Attaching-effacing *E. coli*	1.1.3.2
approx.	Approximately	Table 3-C
BCA	Bicinchoninic acid	2.5.4.2
BFP, *bfp*	Bundle-forming pili, BFP gene cluster	1.1.1
bp	Base-pairs	Table 2-A
BPW	Buffered peptone water	1.4.2.5
CD	Colostrum-deprived	1.4.2.7
CDCD	Caesarean-derived colostrum-deprived	1.2.2
CF	Colostrum-fed	5.1
cfu	Colony-forming unit(s)	1.4.2.7
CNS	Central nervous system	1.3.3.1
CPHL	Central Public Health Laboratory, Colindale, UK	2.2.1
CRGV	Cutaneous and renal glomerular vasculopathy	1.3.3.1E
CT-SMAC	Cefixime-tellurite SMAC	1.4.2.6
DMEM	Dulbecco's modified Eagle's medium	2.2.4A
dpi	Days post inoculation	Table 6-C
eae / eaeA	*E. coli* attaching and effacing gene	1.1.3.1
EAF	EPEC adherence factor	1.1.3.1
EDTA	Ethylene diamine tetraacetic acid	2.3.1
EHEC	Enterohaemorrhagic *E. coli*	1.1.3
EHEC-*hly* / *hly*	EHEC enterohaemolysin gene cluster	1.4.2.4
EIEC	Enteroinvasive *E. coli*	1.1.3
ELISA	Enzyme-linked immunosorbent assay	1.4.2.4
EM	Electron microscope / microscopy	2.7.4.1
eosins	Eosinophils	Table 5-C
EP	Excellent preservation	Table 5-C
EPEC	Enteropathogenic *E. coli*	1.1.3
Esp	EPEC secreted protein	1.2.3.2B
ETEC	Enterotoxigenic *E. coli*	1.1.3
etp	Putative type II secretion system gene cluster	1.4.2.2E
FAS	Fluorescence actin stain / staining	1.2.3.2B
FITC	Fluorescein isothiocyanate	2.5.5.4
Gb_3	Globotriaosylceramide	1.3.3.1A
Glob l'cytes	Globular leucocytes	Table 6-A
Glut	Glutaraldehyde-fixed material	Table 5-C
GP	Good preservation	Table 5-C
gyr	Bacterial gyrase gene cluster	3.7
H&E	Haematoxylin and eosin	2.7.2
HC	Haemorrhagic colitis	1.1.3.1
HIS	Histidine	2.5.4.3B
hpi	Hours post inoculation	Table 5-A
HUS	Haemolytic-uraemic syndrome	1.1.3.1
IMS	Immunomagnetic separation	1.4.2.5
ipx	Immunoperoxidase	2.7.3
IS	Insertion sequence(s)	1.1.1
IVOC	*In vitro* organ culture	1.2.2
katP	Catalase peroxidase gene	1.4.2.2E
kbp	Kilo base-pairs	1.2.3.1
kDa	Kilodaltons	1.2.3.1B
kV	Kilovolts	2.7.4.1
LA	Localised adherence	1.1.3.1

Abbreviation	Meaning	First use (section)
LAgg	Latex agglutination	2.2.10
LB-G	Luria-Bertani minus glucose	2.2.3
LEE	Locus of enterocyte effacement	1.1.3.1
LEP	Laboratory of Enteric Pathogens, CPHL	Table 3-A
Ler	LEE-encoded regulator	1.2.3.1
LM	Light microscope / microscopy	2.7.4.2
LP	Lamina propria	Table 4-D
LPC	Lamina proprial cellularity	Table 5-C
Mbp	Mega base pairs	1.1.1
MC	Microcolony(ies)	Table 3-C
MDa	Megadaltons	1.1.1
mEC	Modified *E. coli* broth	1.4.2.6
min	Minute(s)	2.2.4B
mod	Moderate / moderately	Table 3-C
MW	Molecular weight	Figure 3-b
NAD	No abnormalities detected	Table 4-C
nal, nal^r	Nalidixic acid, nalidixic acid resistant	2.2.9
NCBI	National Center for Biotechnology Information	2.3.2
NCTC	National Collection of Type Cultures	Table 3-A
NM	Non-motile	1.1.2
OD	Optical density	2.5.3
OMP	Outer membrane protein(s)	1.1.1
orf	open reading frame	1.2.3.1
PAGE	Polyacrylamide gel electrophoresis	2.5.4.1
PAP	Peroxidase-antiperoxidase	2.7.3
PBS	Phosphate-buffered saline	2.2.4B
PCR	Polymerase chain reaction	1.4.2.4
Per	Plasmid-encoded regulator	1.1.3.1
PFGE	Pulsed-field gel electrophoresis	1.4.2.6
p.i.	Post-inoculation	1.4.2.7
PLG	Phase-lock gel	2.3.1
PMN	Polymorphonuclear neutrophil(s)	1.3.4.2
PWD	Postweaning diarrhoea	1.1.3.2
RBC	Red blood corpuscles	Table 5-C
REPEC	Rabbit EPEC	1.1.3.2
rif, rif^r	Rifampicin, rifampicin resistant	2.2.9
RpoS, *rpoS*	Sigma 38 stationary phase regulator, RpoS gene	1.4.2.2C
SDS	Sodium dodecyl sulphate	2.3.1
SEM	Scanning electron microscope / microscopy	1.1.3.1
SMAC	Sorbitol MacConkey agar	1.4.2.4
spec, spec^r	Spectinomycin, spectinomycin resistant	2.2.9
STEC	Shiga (like) toxin-producing *E. coli*	1.1.3.1
str, str^r	Streptomycin, streptomycin resistant	2.2.9
Stx	*Shigella dysenteriae* serotype 1 toxin	1.3.1
Stx1(2), *stx1(2)*	*E. coli* Shiga like toxin 1(2), Stx1(2) gene	1.3.1
TBA	Tryptone Bile agar	2.5.1
TBS	Tris-buffered saline	1.4.2.6
TEM	Transmission electron microscope / microscopy	1.2.5
Tir, *tir*	Translocated intimin receptor, Tir gene	1.2.3.1
TSB	Tryptone soya broth	1.4.2.5
TTP	Thrombotic-thrombocytopaenic purpura	1.3.4.2
TVC	Total viable count	2.2.6
V:Cr	Villus:Crypt ratio	Table 5-C
VFA	Volatile fatty acid(s)	1.4.2.2C
VLA	Veterinary Laboratories Agency	2.1.1
VTEC	Verocytotoxigenic *E. coli*	1.1.3.1
WT	Wild-type	Table 3-E

Chapter 1 – GENERAL INTRODUCTION

1.1 *Escherichia coli*

1.1.1 Essential features of Escherichia coli

Escherichia coli (*E. coli*) is a small to medium-sized (2-6 μm long) Gram-negative rod, classified in the family Enterobacteraceae. Enterobacteraceae are facultative anaerobes that ferment sugars to acids. The family includes saprophytes as well as animal and plant parasites, some of which are pathogenic. The normal habitat of *E. coli* is the large intestine and lower small intestine of warm-blooded animals, although survival outside the host is possible for weeks to months. Generic information on *E. coli* has been reviewed by VanDemark and Batzing 1987, Timoney et al. 1988 and Quinn et al. 1994.

E. coli has a typical Gram-negative prokaryote cell wall structure (VanDemark and Batzing 1987). The phospholipid bilayer cytoplasmic membrane is covered externally by cross-linked peptidoglycan with an intervening periplasmic space. External to the peptidoglycan layer is the outer membrane, which also has a phospholipid bilayer structure. Proteins involved in pore formation, transport and adhesion, collectively termed 'outer membrane proteins' (OMP), and lipopolysaccharides are present in the outer membrane. The latter comprise fatty acid chains (lipid A) lying within the membrane and attached to a 'core' polysaccharide at the external surface. Branching polysaccharide chains are attached to this core and extend away from the membrane surface; these form the 'O' (somatic) antigens and are variable between strains but identical over the surface of any one cell. Some *E. coli* strains additionally have a surface polysaccharide capsule.

E. coli has filamentous appendages known as flagella, fimbriae and pili, reviewed by VanDemark and Batzing 1987, Krogfelt 1991 and Smyth et al. 1994. Flagella are approximately 17 nm in diameter and up to 200 μm long. They are helical protein multi-unit structures, which are associated with the cytoplasmic membrane at their base. Their function is to confer motility upon the cell body, which is achieved by rotation of the flagellum. Common usage has tended to prefer the terms 'fimbriae' for adhesive organelles and 'pili' for sexual appendages, although this is not a strictly observed terminology, and the function of many observed appendages is unclear. Pili and fimbriae arise from the cytoplasm or cytoplasmic membrane and genetic encoding of these structures varies between strains. Expression is often dependent on environmental conditions and stage of growth. They are composed of repeating protein subunits and are of 2 to 8 nm diameter and 0.2 to 20 μm length. Type-I fimbriae cause mannose-sensitive haemagglutination and are functionally heterogeneous (Sherman et al. 1985). Morphologically, fimbriae may be divided broadly into

three classes: rigid 7 nm diameter structures, flexible 2-4 nm diameter fibrillar structures, and bundle-forming pili (BFP) which form tangled rope-like structures composed of many 4 to 7 nm diameter fimbriae (Giron et al. 1991; Nataro and Kaper 1998). There is a further class of 2 nm diameter, coiled, fimbrial-type organelles termed 'curli' which bind to tissue matrix proteins (Olsen et al. 1989) and major histocompatibility complex class I molecules (Olsen et al. 1998).

The *E. coli* chromosome is a double-stranded, circular DNA molecule of about 4 mega base pairs (Mbp). In addition to the chromosome, *E. coli* may contain plasmids of circular double-stranded DNA, varying in number from one to 11 and in size from 0.3 to approximately 60 Megadaltons (Mda; Hartl and Dykhuizen 1984). Genes associated with pathogenic subtypes are located on both the chromosome and plasmids (VanDemark and Batzing 1987).

Transfer of genes may occur between plasmids and the chromosome within an *E. coli* cell, between different cells of the same or different strains, or between different bacterial species. Genetic exchange is accomplished by F-pilus conjugation, bacterial virus (bacteriophage) transduction and DNA-uptake transformation (VanDemark and Batzing 1987). Transposable elements are genetic sequences which can move, and sometimes replicate, independently within the genome. These comprise: insertion sequences (IS), transposons and some prophages of bacteriophages (Madigan et al. 1997a). IS encode only the genes required for their mobility, and their locations in the genome may be used to type strains. Transposons encode additional genes which can, for example, include antibiotic resistance-conferring elements. Bacteriophages (phages) may be virulent, where successful infection leads always to production of virus particles and lysis of the bacterium, or temperate, where the viral genome may be integrated into the bacterial genome as a prophage following infection, in a process called lysogeny (Madigan et al. 1997b). Lysogenised bacteria copy the viral prophage with the rest of the bacterial genome during replication. Prophages in lysogenised bacteria may become de-repressed and enter a lytic phase, when mature virus particles are produced and the host cell is lysed. If a prophage becomes damaged, it may lose the ability to enter the lytic phase and will then persist as a defective bacteriophage, possibly encoding genes which are expressed by the bacterium. Prophages may encode certain bacterial virulence factors, for example the Shiga toxin produced by certain strains of *E. coli* (Section 1.3.2).

1.1.2 *E. coli* subclassification schemes of relevance to the present studies

E. coli isolates may be classified and grouped according to diverse features, some of which are related to their capacity to cause disease, as reviewed (Hartl and Dykhuizen 1984;

Nataro and Kaper 1998). Pathogenic strains may be divided into pathotypes according to their associated diseases, as discussed in Section 1.1.3.

Kauffmann (1946) proposed a serological typing system based on surface antigens, utilising heat-stable cell wall ('O'), heat-labile surface and heat-stable capsular ('K'), and flagellar ('H') antigens. Modern serotyping defines the serogroup in terms of the single O antigen and the serotype in terms of the single O and H antigens present on any one isolate. K denotes capsular antigen (usually polysaccharide) when present. Fimbrial ('F') antigens, originally designated 'K', may also be typed when present; in common usage they are often referred to by K number (e.g. K88, K99) or using a designation related to their type strain, e.g. 987P (Krogfelt 1991). Strains without O antigen are designated 'rough' due to their colonial morphology, and strains without flagella are designated 'H-' or 'non-motile'/'NM'. The term 'serovar' refers to any serologically-determined grouping. There is no strong relationship between serotype and genotype, as closely related strains may be of different serotype.

The total genome size of *E. coli* varies by up to 23 % between strains, indicating substantial genetic variation. Gene probes are available for some characteristics associated with virulence, such as Shiga toxin production. Strains may also be genotyped in terms of plasmid content, restriction fragment patterns, IS, phage sequences, metabolic enzyme sequences, and other features of the genome such as strand-biased eight base-pair chromosomal DNA sequences termed octamers (Kim et al. 1999).

E. coli produces several identified exotoxins, allowing typing on the basis of toxin production. Identification of the toxin may be attempted from culture medium or clinical specimens by *in vitro* or *in vivo* assays. For example, Shiga toxin produces a characteristic, progressive shrivelling effect on Vero (African green monkey kidney) cells (Konowalchuk et al. 1977) and HeLa (human cervical carcinoma) cells (O'Brien et al. 1982b). Cytotoxic necrotising factor (CNF) produces a multinucleating cytopathic effect on hamster ovary, Vero and HeLa cells, plus a necrotising lesion when injected intradermally into rabbits (Caprioli et al. 1983). Gene probes for known toxin genes may also be used, and serological evidence of toxin exposure can also be sought (Nataro and Kaper 1998).

Biotypes, biogroups and biovars are distinguished on the basis of metabolic capabilities. Generally these tests have few advantages over other typing techniques, but some tests of high discrimination between closely related strains are in use. Metabolic variation can be of use in the detection and isolation of strains, as in the case of *E. coli* O157:H7 which is distinguished from most other *E. coli* by its inability to ferment sorbitol, allowing the use of sorbitol-containing indicator media to help identify its presence.

The specificity of a particular phage for *E. coli* strains depends on surface properties, and on prior lysogeny of strains with a related phage (Madigan et al. 1997b). A lysogenised

bacterium with, by definition, a repressed phage genome will similarly repress the genome of another phage of the same type if it is introduced into the cell. Phage typing is used in epidemiological research, for example comparing veterinary- and human-derived strains. A bias in the phage types of *E. coli* O157:H7 found in different animal species has been noted (Paiba et al. 2002).

1.1.3 Pathogenic E. coli

E. coli typically are bowel commensals, with the persistence of strains varying over days to months in an individual animal (Hartl and Dykhuizen 1984). Pathotypes are defined in terms of bacteriological and clinical features. Apart from opportunist infections following surface damage or implantation of bacteria into the body, *E. coli* which cause disease possess virulence factors that enable them to colonise mucosal surfaces, evade defence mechanisms, multiply and cause damage (Nataro and Kaper 1998). Virulence attributes identified so far include: a capsule (antiphagocytic), endotoxin (toxic, complement-defeating), fimbriae (adhesive), haemolysins (membrane-damaging, nutrient-releasing), siderophores (iron-binding for growth), exotoxins (variety of effects), attaching-effacing (AE) phenotype (intimate adherence) and acid tolerance (Quinn et al. 1994; Lin et al. 1996). Pathotypes of *E. coli* affecting the gastrointestinal tract of humans include: enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) and enteroaggregative (EAEC, EAggEC), as reviewed by Nataro and Kaper (1998) and Levine (1987). Extra-intestinal infections occur also (Hartl and Dykhuizen 1984). Some strains, for example *E. coli* O157:H7, are pathogenic in one species (Section 1.4.2.3), but are generally considered to be commensal organisms in others (Section 1.4.2.6).

1.1.3.1 *E. coli* O157:H7 and related pathotypes in humans

Enteropathogenic E. coli (EPEC)

Since the original serological definition of EPEC, which were associated with infant summer diarrhoea, other pathotypes have been defined and important virulence factors and pathological mechanisms have been identified. The modern definition of EPEC (Nataro and Kaper 1998) is non-serological and depends on the presence of the AE phenotype *in vitro* (Section 1.2) in the absence of Shiga toxins (Section 1.3).

Clinical features of EPEC diarrhoea have been reviewed (Nataro and Kaper 1998). EPEC are now mainly associated with infant diarrhoea in the developing world. EPEC colonise the small and large intestine (Ulshen and Rollo 1980; Rothbaum et al. 1982) and the hallmark lesion is intimate adhesion to the enterocyte brush border with effacement of microvilli, the

so-called AE lesion (Section 1.2). Initial non-intimate attachment is followed by intimate attachment, with the latter mediated by a cluster of genes called the locus of enterocyte effacement (LEE), which includes the *eaeA* (*E. coli* attaching and effacing) gene encoding intimin (Section 1.2.3.2C).

On HeLa or HEp-2 epithelial cell cultures, both cervical carcinoma lines (Chen 1988), EPEC strains show an initial distinctive, clustering, non-intimate adherence (Scaletsky et al. 1984; Nataro et al. 1985) termed 'localised adherence' (LA). The LA as originally described is seen after a three hour incubation (strong LA), but a second, weak LA pattern is seen with some EPEC only after six hours (Scaletsky et al. 1999). Isolates showing the former pattern are termed 'typical EPEC' and the latter 'atypical EPEC' (Nataro and Kaper 1998). A 50 to 70 MDa plasmid encoding an uncharacterised 'EPEC adherence factor' (EAF) has been described in EPEC which exhibit strong LA (Baldini et al. 1983) and its role in conferring the strong LA phenotype has been confirmed (Levine 1987). An EAF probe derived from this plasmid may be used to discriminate between typical and atypical EPEC. EAF probe sequences have occasionally been found on the chromosome (Nataro et al. 1985). The clinical significance of EAF has been shown in a human volunteer study which demonstrated a significant reduction of the diarrhoeagenic effect in EAF-negative strains derived from control EAF-positive EPEC isolates (Levine et al. 1985). The existence of EAF-positive strains in which strong LA has been abolished following mutations on the chromosome suggests that regulatory chromosomal genes are involved in the LA phenotype (Donnenberg et al. 1990).

The EAF plasmid has been found to contain a gene cluster encoding the production and expression of BFP (Stone et al. 1996). These have been shown to mediate microcolony formation in culture and strong LA on HEp-2 cells, with inter-bacterial and bacterial-host cell connections via BFP evident under scanning electron microscopy (SEM; Giron et al. 1991). The role of BFP *in vivo* is still unclear, as adhesion of EPEC to human duodenal and jejunal organ cultures appeared to be independent of EAF or BFP (Knutton et al. 1991; Hicks et al. 1998). Furthermore, non-intimate adhesion has been shown to require the *eaeA* gene (Hicks et al. 1998). Recently the complete DNA sequence of an EAF plasmid (pB171) was determined (Tobe et al. 1999). It contains operons for BFP production, enhancement of *bfp* gene expression and the Per global regulator (see below). The role of atypical EPEC in diarrhoea is not well defined, but epidemiological evidence for its significance is accumulating (Scaletsky et al. 1999).

The *eaeA* gene, crucial to the full expression of the AE phenotype, has been shown to be an EPEC virulence factor in a human volunteer study (Donnenberg et al. 1993a), however pathogenicity was not completely abolished by the removal of *eaeA*.

An additional property of EPEC, of uncertain significance, is the ability to invade host epithelial cells that has been observed *in vivo* (Fagundes-Neto et al. 1995) and *in vitro* (Miliotis et al. 1989). Invasiveness appears to be influenced by EAF, AE phenotype and other, largely undetermined, attributes. The relationship between *in vitro* cell culture invasion assays and *in vivo* behaviour remains to be clarified (Donnenberg et al. 1990; Fletcher et al. 1992).

A regulatory gene cluster, termed plasmid-encoded regulator (Per) is present on the EAF plasmid. It influences expression of certain genes of the AE phenotype-encoding LEE (Section 1.2.3), including *eaeA*, plus the *bfp* gene cluster (Nataro and Kaper 1998). Per appears to be a global virulence regulator which also may affect host tissue tropism as it has been shown to confer human tropism upon a rabbit *E. coli* pathogen (Tesh and O'Brien 1992).

The diarrhoeagenic mechanisms operating in EPEC infection are not well understood, but observation and experiment have revealed several potentially significant effects occurring in parallel and on varying timescales. These include: increased chloride secretion by enterocytes, increased intestinal permeability associated with decreased transepithelial electrical resistance, maldigestion and malabsorption associated with effacement of microvilli and villus atrophy, and inflammation (Donnenberg et al. 1997).

EPEC strains show more genetic similarity to each other than to other members of their respective serotypes, i.e. there is evidence that EPEC are clonally-derived from a common ancestor (Donnenberg and Kaper 1992). EPEC may be divided into two clonal groups on the basis of multilocus enzyme electrophoresis (Donnenberg and Whittam 2001), which are reflected in the H antigen type (Frankel et al. 1998b) and the chromosomal location of the LEE (Kaper 1998).

Enterohaemorrhagic E. coli (EHEC)

The EHEC pathotype of *E. coli* was identified following two observations in the early 1980s. A distinctive gastrointestinal illness was recognised which was termed haemorrhagic colitis (HC) and which was associated with a previously rare *E. coli* serotype, O157:H7 (Riley et al. 1983). Karmali et al. (1983; 1985) noted the clinical connection between HC, haemolytic-uraemic syndrome (HUS, Section 1.3.4.2), and faecal Shiga (Vero-) toxin or Shiga toxin-producing *E. coli* (STEC, including serotype O157:H7) isolated from stools.

'EHEC' was first defined in 1987 (Levine 1987). The definition currently rests on clinical, phenotypic and genotypic features. 'Typical' EHEC cause HC and HUS, produce Shiga toxins, have an AE phenotype in standard *in vitro* assays and possess a 60 MDa plasmid, termed pO157 or EHEC plasmid. 'Atypical' EHEC lack either or both of the AE phenotype and pO157 plasmid. Important differences from EPEC include: Shiga toxin production, the

absence of EAF/BFP, the nature and variable presence of the AE lesion, and the site of lesions in humans and animal models, this being limited to the large intestine and lower ileum in EHEC infection (Nataro and Kaper 1998).

EHEC are a heterogeneous group, including some non-AE strains (Willshaw et al. 1992), and although the EHEC pathotype is a clinically helpful concept, many workers prefer to use the broader category of STEC (or Verocytotoxigenic *E. coli*, VTEC). This is better-defined when dealing with strains from any source, including non-clinical isolates, and reflects the evidence that Shiga toxin is an essential element in the clinical disorders associated with EHEC.

1.1.3.2 *E. coli* causing enteric disease in the veterinary species

Veterinary pathotypes are classified similarly to human *E. coli*. In particular ETEC (Barker et al. 1993), and EPEC- and EHEC-like strains, are recognised. There is substantial variation in the serotypes causing disease in different species (Taylor 1961), and few human pathogenic serotypes are represented (Nataro and Kaper 1998).

Attaching-effacing E. coli (AEEC), EPEC and EHEC

AEEC not producing Shiga toxin or enterotoxin may be termed EPEC, and have been reported in association with diarrhoea in the calf (Pospischil et al. 1987; Pearson et al. 1989; Janke et al. 1990), dog, (Broes et al. 1988; Drolet et al. 1994a), rabbit, pig, lamb (Barker et al. 1993; Finlay and Abe 1998), goat (Drolet et al. 1994b) and cat (Pospischil et al. 1987).

Rabbit EPEC (REPEC) have been extensively studied. REPEC have been found in several serogroups, and two subtypes have been identified. One affects suckling rabbits, with AE lesions throughout the intestinal tract, and the other affects weanling rabbits, with AE lesions restricted to the lower ileum and large intestine (Cantey and Blake 1977; Prescott 1978). The weanling subtype appears to be of low virulence in the neonate (Peeters et al. 1984a) and vice versa (Peeters et al. 1984b). RDEC-1 is a REPEC affecting weanlings and older rabbits, expressing a plasmid-encoded fimbrial adhesin (AF/R1; Berendson et al. 1983) which mediates specific non-intimate adherence to the rabbit intestinal brush border (Sherman et al. 1985; Wolf et al. 1988), and which increases virulence and AE lesion formation *in vivo* (Wolf et al. 1988). Another fimbrial adhesin, AF/R2, is expressed by REPEC O103 (Nougayrede et al. 1999). A gene cluster homologous with the LEE of human EPEC has been found in RDEC-1 (Deibel et al. 1998).

AEEC are isolated frequently from both healthy and sick calves, and there is evidence of a particular association between calf diarrhoea and the presence of the beta subtype of intimin (China et al. 1998; China et al. 1999). In several reports, EPEC-like organisms have been associated with AE lesions in the large and small intestines of diarrhoeic calves (Janke

et al. 1989; Janke et al. 1990; Holland et al. 1999), although only in one case has a positive identification of the organism *in situ* been made (Pearson et al. 1989). A natural HC-like disease in calves has been reported in association with several *E. coli* serotypes (Hall et al. 1985; Schoonderwoerd et al. 1988; Johnson et al. 1996), some of which have been shown to produce AE lesions experimentally in calves (Chanter et al. 1984; Moxley and Francis 1986; Schoonderwoerd et al. 1988; Wray et al. 1989) and to be Shiga toxin-producers (Moxley and Francis 1986; Hall et al. 1988; Schoonderwoerd et al. 1988; Wray et al. 1989). Diagnostic investigations have revealed AE lesions associated with STEC, including O26:H11, in diarrhoeic calves with and without dysentery (Iijima et al. 1990; Janke et al. 1990; Gunning et al. 2001). Isolates of *E. coli* O26:H11 hybridising with *eaeA* and EHEC plasmid probes but lacking Shiga toxin have also been isolated from diarrhoeic calves (Saridakis et al. 1997). Haemorrhagic diarrhoea with AE lesions has been reported in an adult cow (Wada et al. 1994) and an eight-month old heifer (Pearson et al. 1999). In the latter, Shiga toxin and other EHEC genes were detected in an associated O26:H11 strain isolated from a symptomatic in-contact animal. In summary, bovine EHEC-type strains causing diarrhoeal disease have been identified but there is significant variation in the virulence attributes identified between strains and information is patchy. The human EHEC serotypes O26:H11 and O111:H- have been implicated, but not O157:H7. No HUS-like disease is reported in calves, but this may be difficult to identify in the field, as dehydration and acidosis of diarrhoeic calves may obscure and preclude assessment of haematological abnormalities.

AE lesions have been seen in a few cases of postweaning diarrhoea in pigs (Section 1.1.3.2), associated with *E. coli* (Janke et al. 1989), including serogroup O45 (Helie et al. 1991). Experimentally, *E. coli* O116 has been associated with AE lesions in weaned pigs (Neef et al. 1994). In pigs and sheep, Shiga toxin-producing *E. coli* (STEC) have not been associated with AE lesions *in vivo*, and no association with haemorrhagic colitis has been made. However, a colostrum-deprived lamb inoculated orally with a bovine EHEC strain did develop dysentery and die (Moxley and Francis 1986).

Oedema disease and postweaning diarrhoea in pigs

Oedema disease of swine is associated with *E. coli* producing a subtype of Shiga toxin (Stx2e) which causes a systemic angiopathy leading to death from vascular leakage. Outbreaks are often associated with postweaning diarrhoea (PWD) within the affected group. Oedema disease and PWD strains occur in serogroups O138, O139, and O141, plus O149 in some cases of PWD. Some oedema disease strains produce an enterotoxin. PWD strains are enterotoxigenic and some additionally produce Shiga toxin. The F18 fimbrial adhesin is implicated in colonisation of the small intestine in both diseases (Gyles 1998), but typically AE lesions are not found.

1.2 The Attaching-Effacing (AE) Lesion

1.2.1 Morphology

The AE lesion is characterised by intimate adherence between the bacterium and the epithelial cell membrane with an intervening gap of about 10 nm, plus effacement of enterocyte microvilli. The lesion was first described in rabbits (Polotsky et al. 1977; Takeuchi et al. 1978). Beneath the adherent bacterium a cytoskeletal rearrangement, including the accumulation of filamentous actin (F-actin), is seen. The bacteria often sit upon a pedestal-like structure, which can extend up to 10 μm away from the epithelial cell surface (Kaper et al. 1998).

1.2.2 Animal and in vitro models

Young rabbits and gnotobiotic or caesarean-derived colostrum-deprived (CDCD) piglets have proved convenient and useful models for studying AE lesions. They are susceptible to human AEEC and, in the case of the rabbit, there are well-characterised AEEC adapted to the model species. Piglets are usually inoculated at one to two days of age and develop AE lesions in the distal ileum and the large intestine when inoculated with EPEC, EHEC, bovine EHEC-like and REPEC strains (Moon et al. 1983; Tzipori et al. 1985; Hall et al. 1988; Tzipori et al. 1989). The susceptibility of piglets to AE lesions appears to decline with increasing age (Moon et al. 1983).

Rabbits exhibit AE lesions with REPEC, EPEC and EHEC (Moon et al. 1983; Sherman et al. 1988a), and EPEC-induced AE lesions have been observed with *in vitro* organ culture (IVOC) of the rabbit ileum (Batt et al. 1987).

Studies in mice with EHEC have not revealed AE lesions (Wadolkowski et al. 1990), but *Citrobacter rodentium*, which encodes a LEE, does induce AE lesions in murine transmissible colonic hyperplasia (Adu-Bobie et al. 1998).

IVOC using AEEC-inoculated human tissues has shown AE lesions (Knutton et al. 1987; Phillips and Frankel 2000a; Phillips et al. 2000b; Fitzhenry et al. 2002). Common human cell lines on which AE lesions are formed include HEp-2, HeLa, Henle (Int) 407 (all cervical carcinoma derivatives; Gey et al. 1952; Lavappa 1978; Chen 1988), Caco-2 (colon carcinoma), T84 (colon), HCT-8 (ileocaecum) and HEL 229 (human embryonic lung fibroblast) (Knutton et al. 1989; Francis et al. 1991; Tesh and O'Brien 1992; McKee and O'Brien 1995b).

1.2.3 Mechanisms

1.2.3.1 The locus of enterocyte effacement (LEE)

The LEE pathogenicity island has been found in all tested bacterial strains which express the AE phenotype (McDaniel et al. 1995). In EPEC O127:H6 It is a 35.6 kbp chromosomal insertion, encoding 41 predicted open reading frames (orf), which have a distinct C and G nucleotide percentage (38.3 %) when compared with the rest of the *E. coli* genome (50.8 %), indicating an origin outside the species (Elliott et al. 1998). The EPEC LEE is necessary and sufficient for expression of the AE phenotype *in vitro* (McDaniel and Kaper 1997), but the EHEC O157:H7 LEE is larger (approximately 41 kbp) and appears to be insufficient on its own for that function (Section 1.4.2.2B; Elliott et al. 1999). Differences between the LEE genes of EHEC and EPEC are most marked among the proteins which interact directly with the host (Perna et al. 1998), which possibly contributes to the different disease patterns observed.

For both EPEC and EHEC there are four operons (LEE 1-4) which encode secreted proteins and a type III secretion apparatus (Section 1.2.3.2B, part ii), plus an operon (Tir) encoding the Tir and intimin proteins (Section 1.2.3.2C). Within LEE 1 is an orf termed Ler (LEE-encoded regulator) which is under the positive influence of the Per regulator found on the EAF plasmid of EPEC, and which upregulates expression of LEE operons 2 to 4 (Mellies et al. 1999). Additionally, Ler promotes expression of the Tir operon and of other non-LEE secreted proteins of EPEC and EHEC, suppresses novel fimbriae in EPEC and EHEC, and alters adherence patterns *in vitro* (Elliott et al. 2000). It therefore appears to act as a global regulator.

Investigations into regulation mechanisms have revealed that the EPEC and EHEC LEE, including Ler, is under the influence of a quorum-sensing mechanism, mediated by the autoinducer product of the *luxS* gene which is found in many *E. coli* and is released into supernatant (Sperandio et al. 1999). It has been hypothesised that quorum sensing is to some extent responsible for the upregulation of virulence determinants, such as the LEE, in an intestinal environment, using signals from the intestinal flora (Sperandio et al. 2001). EPEC, which colonise the relatively bacteria-poor small intestine, may compensate for the low signal from the surrounding flora via the Per promoter (Sperandio et al. 1999), whereas this may be an unnecessary mechanism for EHEC, which lack Per and which colonise the bacteria-rich large intestine. In addition, another quorum sensing system, involving the SdiA regulator, appears to act as a late-stage repressor of LEE expression in EHEC O157:H7, but not in EPEC (Kanamaru et al. 2000).

There is one report of a plasmid-borne REPEC AE determinant (Fletcher et al. 1990). It was not possible to identify any LEE genes on subsequent investigation of the plasmid, nor

was any single gene from the plasmid able to confer the AE phenotype (Fletcher et al. 1992). The possibility of a plasmid-borne regulatory gene cluster could not be excluded and the presence of a chromosomal LEE was not determined.

1.2.3.2 Development of the AE lesion

A three-stage model for the AE process has been proposed (Donnenberg et al. 1997), although a final invasion stage may be included (Tesh and O'Brien 1992).

A. Initial non-intimate attachment

An early non-intimate attachment appears to be necessary for initial signalling leading to development of the lesion, but the mediators of this attachment are poorly understood. Studies have shown a potentiating effect of suspected adhesive factors, such as EAF and the REPEC-associated AF/R1, on AE lesion formation *in vitro* and on AEEC virulence *in vivo*. Several putative *E. coli* O157:H7 adhesins have been identified *in vitro* (Section 1.4.2.2D), but their roles in promoting AE lesion formation are unknown. EspA filaments (Section 1.2.3.2B) may act as an adhesin for EPEC (Knutton et al. 1998; Daniell et al. 2001b) and for *E. coli* O157:H7 (Tatsuno et al. 2000). Intimin and Tir, which are directly involved in intimate attachment, appear to be important for the primary adhesion and microcolony formation of *E. coli* O157:H7 (Section 1.4.2) *in vitro* and for detectable adherence *in vivo* (Marches et al. 2000), but intimin-deficient mutants may still adhere diffusely *in vitro* (Tatsuno et al. 2000).

B. Signal transduction leading to cytoskeletal reorganisation and microvillus effacement.

AEEC use a type III secretory apparatus, which functions to deliver bacterial proteins into the cytosol of the host eukaryotic cell (Hueck 1998). The type III apparatus is encoded on the LEE by *sep* and *esc* genes (Elliott et al. 1998), and another LEE gene (*pas*) is necessary for its function in EHEC O157:H7 (Kresse et al. 1998). The LEE encodes several proteins secreted by the type III apparatus, termed EPEC-secreted proteins (Esp), which are induced in eukaryotic cell culture medium and *in vivo*. Three Esp (A, B and D) are essential for normal AE lesion formation. EspA forms filaments on the bacterial surface, in an EspD-dependent manner (Knutton et al. 1998), which adhere to host cells (Shaw et al. 2002). There is evidence that EspD participates in forming pores in the host cell membrane also (Daniell et al. 2001a). EspD from an O157:H7 EHEC has been localised to the membrane of the target cell (Kresse et al. 1999) and certain AE diffusely-adhering *E. coli* (Nataro and Kaper 1998) insert EspD into the host cell membrane (Wachter et al. 1999). EspB is translocated to the host cell in an EspA-dependent manner (Ebel et al. 1998; Knutton et al. 1998), and is

distributed in the plasma membrane and the cytosol of the host cell (Wolff et al. 1998). Although EspB binds EspA and is co-localised with it, EspB is not necessary for EspA interaction with host cells (Hartland et al. 2000). An Esp -A, -B and -D -dependent haemolytic action of EPEC has been reported, suggesting that all three of these proteins may be involved in pore formation in eukaryotic cell membranes (Warawa et al. 1999).

A current model proposes that a bacterial transmembrane structure, encoded by *escC,* links with a hollow EspA filament to form a bi-functional organelle which has adhesive and protein-translocating roles (Daniell et al. 2001b). Esp -B and -D may create a eukaryotic cell membrane pore-forming structure. Connected together, these elements could form a molecular 'syringe' for the introduction of bacterial macromolecules into the host cytosol (Frankel et al. 1998b). The distribution of EspB within the host cell membrane plus cytosol (Taylor et al. 1998; Wolff et al. 1998) and the importance of EspB in the phosphorylation of Tir (see below) suggests that EspB may have other functions instead of, or in addition to, that proposed. Significant differences in the roles of the Esp proteins of EPEC and EHEC are becoming apparent, with the adhesion of mutants of EHEC O157 in EspA and EspD being more profoundly affected than that of similar mutants of EPEC (Kresse et al. 1999).

Two secreted proteins, EspC (110 kDa) and EspF (21 kDa) are not essential for the AE phenotype (Donnenberg et al. 1997; Frankel et al. 1998b). EspF appears to have a role in disrupting the intestinal epithelial barrier function (McNamara et al. 2001), and EspC is secreted independently of the type III mechanism and has an enterotoxic activity (Mellies et al. 2001).

AEEC translocate a LEE-encoded 72 to 80 kDa receptor for the *eaeA* gene product (intimin), termed the translocated intimin receptor (Tir), into the host cell (Rosenshine et al. 1992; Kenny et al. 1997b; Deibel et al. 1998; DeVinney et al. 1999). This translocation is dependent on the type III secretion system. Tir undergoes an apparent increase in molecular mass within the host cell, from 72 kDa to 88 kDa in the case of *E. coli* O157:H7 (DeVinney et al. 1999). With EPEC O127:H6 the shift is from 78 kDa to 85 kDa and involves serine residue phosphorylation (Warawa and Kenny 2001). The alteration in apparent mass may be due to conformational changes, which may in turn be required for a functional conformation within the host cell membrane (Warawa and Kenny 2001). Tir is thought to adopt a hairpin-loop conformation when *in situ* in the host cell membrane, with the C- and N-terminals located on the cytoplasmic side of the plasma membrane and an externally-presented loop forming the Tir-intimin binding area (Hartland et al. 1999; Kenny 1999).

Kenny (1999) has shown that additional phosphorylation of tyrosine 434 of EPEC Tir is required for filamentous actin focusing (see below), but this modification is not required for membrane insertion and does not contribute to shifts in apparent molecular mass. Tyrosine phosphorylation does not occur with *E. coli* O157:H7 Tir (DeVinney et al. 1999), where

tyrosine 434 is replaced by serine (Kenny 1999), but the molecule remains associated with filamentous actin nucleation. Phosphorylation of proteins at the site of the lesion appears to be dependent upon EspB secretion (Ismaili et al. 1998).

At the site of intimate attachment, considerable eukaryotic cytoskeletal reorganisation occurs, with depolymerisation of actin, the formation of filamentous actin (F-actin) and the accumulation of α-actinin, myosin light chain, talin and ezrin (Donnenberg et al. 1997). α-actinin cross-links actin, and talin and ezrin are believed to link actin with transmembrane proteins such as integrins (Finlay et al. 1992). These cytoskeletal changes are associated with effacement of microvilli and disruption of the intestinal barrier function of enterocytes (Simonovic et al. 2001). The F-actin may be detected *in vitro* by virtue of its specific affinity with the fungal toxin phalloidin, or by antibody labelling. Fluorescein-conjugated phalloidin forms the basis of the filamentous actin staining (FAS) test, by which AE lesions may be detected and characterised *in vitro* (Knutton et al. 1989) on a variety of cell lines, including HeLa, HEp-2, Caco-2, and HEL 229 (human embryonic lung fibroblast).

The mechanisms by which cytoskeletal elements are dissolved and reaggregated are unclear. Current theories implicate translocated bacterial proteins such as EspB and Tir, which are co-localised at the site of cytoskeletal reorganisation, plus activation of host cell protein kinase activity (Frankel et al. 1998b).

C. Intimate attachment

Intimin is a 94 kDa bacterial surface-exposed outer membrane protein which is the product of the *eaeA* gene (Jerse and Kaper 1991; Louie et al. 1993). Tir binds intimin (Rosenshine et al. 1996; DeVinney et al. 1999; Hartland et al. 1999); both proteins are necessary for the development of the mature AE lesion (Donnenberg et al. 1993b; Rosenshine et al. 1996; Kenny et al. 1997b) and both are virulence factors for REPEC *in vivo* (Marches et al. 2000). Examination of *eaeA*-negative mutants *in vitro* reveals a 'shadow' FAS phenotype demonstrating a dim, diffuse FAS reaction (Jerse et al. 1990), indicating that intimin is required for intimate adhesion but is not necessary for the earlier stages of signal transduction. Cells bearing EPEC O127:H6 Tir appear to form normal AE lesions with EHEC O157:H7, but AE lesions between EPEC O127:H6 and cells bearing EHEC Tir are attenuated (DeVinney et al. 1999). This indicates that there is a limited degree of functional interchangeability between intimin and Tir from different pathotypes of *E. coli*.

Intimin/*eaeA* has protein and gene sequence homology with the invasin molecule which promotes invasion of host cells by *Yersinia* (Isberg and Leong 1990), but it is significantly different in the protein C-terminal domain, which is the part responsible for binding to host cells (Frankel et al. 1994). Five principal subtypes of intimin (α, β, γ, δ and ε) are currently recognised, based on antigenic and amino acid sequence differences at the C-terminal

domain (Adu-Bobie et al. 1998; Oswald et al. 2000). The occurrence of intimin subtypes parallels the clonal groupings established for EPEC and EHEC. Thus, most EPEC express either α-intimin (clonal group 1) or β-intimin (clonal group 2), and most EHEC express either β-intimin (clonal group 2) or γ-intimin (clonal group 1, including O157:H7).

A Tir-independent c-lectin-like binding site has been found in the C-terminal domain of intimin, which has been shown to be necessary for expression of the AE phenotype (Frankel et al. 1998a; Hartland et al. 1999). Speculation that intimin subtype may confer tissue tropism upon AEEC, reflected in differing zones of gut affected by EPEC and EHEC (Tzipori et al. 1995) was discounted following the observation that EPEC Tir binds multiple intimin subtypes (Hartland et al. 1999). The finding of another binding site on intimin which is necessary for AE lesions once again raises this possibility, although detailed analysis of EPEC intimin has shown that binding of c-lectins by intimin in the conventional manner is unlikely (Luo et al. 2000). Experiments using piglets and EHEC *eaeA*-negative mutants complemented with EPEC and EHEC *eaeA* genes have produced conflicting results. In one experiment, EPEC *eaeA* in EHEC O157:H7 produced an EPEC-type (distal half of the small intestine plus large intestine) lesion distribution (Tzipori et al. 1995), whilst in another, a similar manipulation produced an EHEC-type (large intestine and occasionally terminal ileum) distribution (Donnenberg et al. 1993b).

Intimin has been shown also to bind β_1-integrins (Frankel et al. 1996). The significance of this is unknown, as intimin-bearing EPEC cannot bind intimately to host cells in the absence of Tir (Rosenshine et al. 1996).

D. Invasion

The tendency for EPEC invasion of host cells is promoted by EAF and *eaeA* (Francis et al. 1991), plus other unknown determinants (Fletcher et al. 1992). EHEC show less tendency to invade *in vitro* but co-incubation with EPEC increases the invasiveness of O157:H7 on HEp-2 cells (Ismaili et al. 1998).

1.2.4 Attaching-effacing lesions in clinical specimens

1.2.4.1 Human

AE lesions have been reported in intestinal tissues from several diarrhoeic infants (Ulshen and Rollo 1980; Rothbaum et al. 1983). Despite forming AE lesions *in vitro*, *E. coli* O157:H7 has not yet been observed to cause AE lesions *in vivo* in human intestine.

1.2.4.2 Veterinary species

AE lesions were first illustrated, incidentally, in piglets inoculated with a human EPEC serotype (Staley et al. 1969). Field cases of AE lesions have been reported or illustrated from veterinary species, initially in rabbits in 1978 (Takeuchi et al. 1978), and subsequently in calves (Moon et al. 1979; Hall et al. 1985), cats (Pospischil et al. 1987), lambs, pigs and young dogs (Janke et al. 1989), goats (Drolet et al. 1994b) and chickens (Fukui et al. 1995).

1.2.5 Detection

FAS is considered to be a definitive test for AE lesions *in vitro*, but adhesion characteristics of AEEC vary, particularly among EHEC (Karch et al. 1987; Knutton et al. 1989; Tesh and O'Brien 1992). AEEC which do not adhere to the particular test cell line may give a false negative result. Amongst animal pathogens the sensitivity of assays on commonly-used cell lines may be low. For example, REPEC O103 is AE and FAS-negative on HeLa cells, but FAS-positive on a rabbit cell line (Nougayrede et al. 1999), and bovine LEE-positive STEC are more commonly FAS-positive on a bovine cell line than on HEp-2 cells (Wieler et al. 1998). Interpretation of the FAS test requires care as there is a 'shadow', unfocussed, lesion which occurs when signal transduction is not followed by full lesion formation (Jerse et al. 1990).

The characteristic morphology of AE lesions may be seen using transmission electron microscopy (TEM) on intestine or cell culture sections.

1.3 Shiga Toxins

1.3.1 Introduction

A parallel terminology exists with Shiga toxin nomenclature, relating to source (*Shigella*) and effect (Verocytotoxicity). The following terminology and abbreviations will be used in the present thesis:

'Shiga toxin'	meaning any of the Shiga toxin family (Shiga toxin, Shiga-like toxin)
'Stx'	meaning *Shigella dysenteriae* serotype 1 toxin
'Stx1'	meaning *E. coli* Shiga (like) toxin 1 / Verotoxin 1 / VT1
'Stx2'	meaning *E. coli* Shiga (like) toxin 2 / Verotoxin 2 / VT2
'STEC'	meaning Shiga (like) toxin-producing *E. coli* / Verocytotoxin-producing *E. coli* / VTEC

Konowalchuck et al. (1977) were the first to report an *E. coli* toxin or toxins lethal to Vero cells and distinct from enterotoxin. These toxins are related to Stx, and two antigenically non cross-reactive groups, Stx/Stx1 and Stx2, are recognised (Scotland et al. 1985). Both are cytotoxic for Vero and HeLa cells (Griffin and Tauxe 1991).

1.3.2 Shiga toxin structure, subtypes and genetics in E. coli

Shiga toxins have a subunit structure consisting of a single A subunit plus five B subunits (Melton-Celsa and O'Brien 1998a). Stx1 is neutralised by anti-Stx antiserum (O'Brien et al. 1982b), appears to be antigenically homologous to Stx and differs from it by one amino acid in the A subunit (Strockbine et al. 1988). Expression of both Stx and Stx1 is regulated by environmental factors including temperature and the presence of iron (O'Loughlin and Robins-Browne 2001). Stx2 comprises an antigenically related family of subtypes including Stx2, Stx2c, Stx2d and Stx2e which are not neutralised by anti-Stx/Stx1 antiserum (Marques et al. 1986) and which apparently are not environmentally regulated (O'Loughlin and Robins-Browne 2001). The genes encoding Stx1 and Stx2 (*stx1* and *stx2*) are chromosomally located and are inserted by temperate toxin-converting bacteriophages (Scotland et al. 1983; Williams Smith et al. 1983; O'Brien et al. 1984; Strockbine et al. 1986), with the exception of Stx2e (Williams Smith et al. 1983; O'Brien and Holmes 1987). Shiga toxin production may occur at a late stage in the lytic phase of the bacteriophage cycle (Plunkett et al. 1999), but some Shiga toxin genes may be associated with defective bacteriophages (O'Brien et al. 1989). STEC may produce either or both Shiga toxin subtypes and multiple copies of *stx2* are found in some STEC (Lindgren et al. 1993). Stx2 cytotoxicity appears to be more strongly associated with culture supernatant than Stx1, which is more cell-associated and is released in larger amounts following lysis of bacterial cultures (Nataro and Kaper 1998).

Shiga toxin production is usually assayed by antibody neutralisation of the characteristic cytotoxicity on HeLa or Vero cells. This technique has demonstrated high, medium and low degrees of cytotoxicity, with neutralising titres varying by up to ten thousand-fold (O'Brien et al. 1982b; Hall et al. 1988) and which appear to be related to the amount of Shiga toxin produced (O'Brien and Laveck 1982a). *E. coli* demonstrating low-level cytotoxicity on cell assays include RDEC-1, some ETEC and EIEC and the laboratory strain K-12 (O'Brien et al. 1982b; Marques et al. 1986). Such cytotoxicity appeared to be entirely bacterial cell-associated in one study (Marques et al. 1986). When probed for Shiga toxin genes, low-level Verocytotoxic *E. coli* are found to be negative, and are now considered not to be STEC (Nataro and Kaper 1998). Some non-O157:H7 *E. coli* strains associated with haemorrhagic colitis were found to be low level Shiga toxin producers (Marques et al. 1986), although the possibility of concurrent infection with undetected high-level STEC in these clinical cases

cannot be excluded. Strains which contain Shiga toxin genes and which do not produce detectable Shiga toxin have been found (Bonardi et al. 1999).

1.3.3 Mechanisms of toxicity and target tissues

1.3.3.1 Animal models

A. Rabbit

Oral inoculation of infant rabbits with EHEC O157:H7 produced diarrhoea with AE lesions in the large intestine. Oral dosing with cell-free Shiga toxin extracts from the same strain produced colitis with diarrhoea (haemorrhagic in one animal) without AE lesions (Pai et al. 1986). Histology revealed widespread epithelial apoptosis in the colon. Thus, haemorrhagic colitis was shown to be induced by Shiga toxin without AE lesions in this case. A dilating effect of Shiga toxin in ileal loops has been demonstrated in rabbits (Williams Smith et al. 1983). Intravenous inoculation of Stx1 produces non-bloody diarrhoea and neurological signs in adult rabbits (Richardson et al. 1992), associated with localisation of Stx1 in the intestinal and central nervous system (CNS) microvasculature. Intravenous inoculation of STEC culture supernatants, including Stx2e-like toxin, induced caecal oedema and paralysis (Williams Smith et al. 1983). Adding the *stx1* gene to RDEC-1 potentiated the diarrhoeagenic effect of the pathogen (Sjogren et al. 1994). In the rabbit, therefore, Shiga toxin has an intrinsic diarrhoeagenic effect by oral and parenteral routes. Shiga toxin globotriaosylceramide receptors (Gb_3, Section 1.3.3.2) have been detected at the rabbit intestinal brush border, but not before 16 days of age (Mobassaleh et al. 1988). The secretory (gut loop dilating) response to Shiga toxin parallels the development of Gb_3 in this location.

B. Pig

In contrast to the rabbit, reports concerning piglets do not show any diarrhoea-potentiating (Tzipori et al. 1987) or ileal loop-dilating (Williams Smith et al. 1983) effects of Shiga toxin. Oedema of the mesocolon was noted in piglets experimentally infected with STEC O157:H7, and intravenous inoculation of older pigs with an Stx2e-like Shiga toxin produced oedema disease-like effects (Williams Smith et al. 1983). Neurological signs and CNS pathology have been reported in orally inoculated piglets using *eaeA*-negative STEC (Tzipori et al. 1995, also Section 1.4.2.7).

C. Mouse

Parenteral (intravenous or intraperitoneal) administration of Shiga toxin has produced a variety of effects. Renal tubular necrosis, epithelial or full-thickness necrosis of the colon, intestinal vascular damage, hindlimb paralysis and death have been reported (Williams

Smith et al. 1983; Beery et al. 1984). Stx2 has a far lower lethal dose than Stx1. An oral infection model has been developed using streptomycin-resistant STEC in animals, in which the commensal gut flora is suppressed by streptomycin treatment. The ability to colonise the murine distal small intestine and to grow in small intestinal mucus were found to be important for virulence in this model (Wadolkowski et al. 1990; Lindgren et al. 1993). Lethality is apparently due to Stx2-induced renal tubular necrosis, and colonic mucosal lesions are mild or absent (Lindgren et al. 1993). Stx2d is activated by murine or human colonic mucus, and strains producing this subtype are far more virulent than other STEC in mice (Melton-Celsa et al. 1998b). The streptomycin-treated mouse model has also demonstrated an acute, fatal encephalomyelopathy associated with damage to CNS myelin sheaths and vasculature (Fujii et al. 1994).

D. Baboon

Intravenous administration of Stx1 to baboons induced necrosis of the intestinal mucosal epithelium and the renal proximal tubule epithelium. Renal glomerular cell swelling and detachment plus thrombosis were observed. Myelin sheath damage was observed in the CNS. Clinicopathological changes reminiscent of HUS were observed (Taylor et al. 1999).

E. Greyhound

There is a striking similarity between HUS and a condition of greyhounds associated with STEC called idiopathic cutaneous and renal glomerular vasculopathy (CRGV; Fenwick and Cowan 1998), but reports are limited.

1.3.3.2 Current understanding

The Shiga toxin B subunits form a pentamer (Melton-Celsa and O'Brien 1998a) which binds a eukaryotic glycolipid receptor which is typically Gb_3, but in the case of Stx2e it is globotetraosylceramide (Gb_4; Gyles 1998). Following endocytosis of the toxin and receptor, the A subunit is enzymatically nicked, releasing an A1 subunit which binds the 60S ribosome and inhibits protein synthesis (Melton-Celsa and O'Brien 1998a). Other receptors or binding sites may also be involved (Cooling et al. 1998; Devenish et al. 1998). The varying distribution of Gb_3 between tissues and species is believed to explain some of the differences in susceptibility and lesion patterns. The human microvascular endothelium, particularly in the intestine and kidney, is sensitive to Shiga toxin (Louise and Obrig 1995; Ohmi et al. 1998; Jacewicz et al. 1999). Murine renal tubules are targeted, in contrast to human renal tissue where glomeruli are affected (Lindgren et al. 1993). In the baboon, both tubular and glomerular renal tissues are affected (Taylor et al. 1999).

1.3.4 Clinical entities associated with STEC

1.3.4.1 Haemorrhagic colitis

The clinical features of HC are described in Section 1.4.1. An epidemiological association between STEC and HC was first made in 1983 (Karmali et al. 1983). A human volunteer study using Stx-positive and negative *Shigella dysenteriae* 1 found that the Stx-negative strain induced less severe dysentery (O'Brien and Holmes 1987). This evidence plus clinical histopathology and the fact that human enterocytes lack Gb_3 receptors (O'Loughlin and Robins-Browne 2001) supports the involvement of a Shiga toxin-induced vasculopathy in HC.

1.3.4.2 Haemolytic-uraemic syndrome (HUS) and Thrombotic-thrombocytopaenic purpura (TTP)

HUS was first described in 1955 and is defined by the triad of microangiopathic haemolytic anaemia, renal failure and thrombocytopaenia. It is associated with intravascular platelet and fibrin thrombosis, particularly in the kidney. Patients with TTP additionally show fever and neurological signs and have a wider distribution of thrombi in organs, although the renal signs may be less severe (Morrison et al. 1986; Griffin and Tauxe 1991). Either may be caused by a variety of factors, but the principal association currently is with EHEC infection in children and the elderly, following the acute, diarrhoeic phase of the illness (Griffin and Tauxe 1991).

The association between HUS and EHEC infection (Karmali et al. 1983; Karmali et al. 1985), reversed a previous belief that HUS caused gastrointestinal signs (Whitington et al. 1979). Evidence for the causal link between Shiga toxin and HUS/TTP includes the observation that the only bacteria-associated HUS involves Shiga toxin-producing organisms (including *Shigella dysenteriae* 1) and the observed sensitivity of the human microvascular endothelium to Stx2, particularly in the target organ of the kidney (Louise and Obrig 1995). A risk factor for HUS in EHEC infection is the presence of Stx2, particularly if it is not accompanied by Stx1. There is good evidence that, in humans, circulating Shiga toxin is bound to polymorphonuclear neutrophils (PMN) and this is a primary route of delivery of the toxin to target organs (Loo et al. 2001).

HUS/TTP is thought to result from systemic Shiga toxin damage to the vascular endothelium, but additional factors are probably involved, such as the binding of Shiga toxin to platelets (Cooling et al. 1998). Some cell cultures lack Gb receptors and are insensitive to the effects of Shiga toxin, but nonetheless do internalise and transport the toxin, suggesting a possible enterocyte-mediated route of delivery of the toxin from the intestinal lumen into the circulation (O'Loughlin and Robins-Browne 2001).

1.3.4.3 Oedema disease of swine

Oedema disease (Section 1.1.3.2) is associated with Stx2e STEC (Gyles 1998) and an angiotoxic effect of the toxin seems likely. Stx2e is frequently found in STEC from cattle (Suthienkul et al. 1990), but a disease association has not been reported in this species.

1.3.4.4 Idiopathic cutaneous and renal glomerular vasculopathy

Associations have been made between CRGV (Section 1.3.3.1E) and STEC in the diet and faeces, faecal Shiga toxin and diarrhoea. Injected Shiga toxin has produced similar clinical effects in greyhounds (Fenwick and Cowan 1998).

1.3.5 Occurrence of STEC in veterinary species and food

STEC are isolated frequently from ruminants, ranging from 1 to 45 % of animals tested (Mainil et al. 1987; Blanco et al. 1988; Johnson et al. 1996), with a wide geographical variation. Isolation rates are higher from calves than from adult cattle (Wells et al. 1991; Cobbold and Desmarchelier 2000). Excretion of STEC by a group of feedlot cattle was shown to vary markedly between sampling occasions, suggesting 'spikes' of high prevalence between longer periods of much lower prevalence (Midgley et al. 1999). Excretion of STEC by sheep was found to be persistent in one study (Asakura et al. 1998). An association between bovine STEC and *eaeA* or the AE phenotype has been reported from several studies (Wray et al. 1993; Asakura et al. 1998; Ismaili et al. 1998; Wieler et al. 1998), although most veterinary and food STEC isolates are LEE-negative (Kaper 1998). Serogroups (Wray et al. 1993; Ismaili et al. 1998) and serotypes (Blanco et al. 1993; Hsia et al. 1993) associated with human EHEC infection are found frequently in animals. There is evidence for a higher rate of STEC excretion among animals at slaughterhouses (Suthienkul et al. 1990). An association between STEC and calf diarrhoea has been suggested by one study (Mohammad et al. 1985) but not supported by others (Blanco et al. 1993; Holland et al. 1999). STEC of EHEC serotypes are found frequently in calves (Blanco et al. 1988; Griffin and Tauxe 1991). Monogastric species have a 'low' isolation rate of STEC, below 10 % and often below 1 % (Johnson et al. 1996).

The preponderance of STEC in young ruminants is reflected in isolations from meat, with STEC found in 15 to 40 % of minced beef samples in Canadian studies, 63 % of veal and lamb samples in a Seattle study, and 17 % of minced beef samples in the UK. Studies in continental Europe have shown a lower isolation rate, up to approximately 6 % (Johnson et al. 1996).

1.4 Enterohaemorrhagic *E. coli*

1.4.1 Human clinical features

Haemorrhagic colitis typically has a 3- to 4-day incubation followed by crampy abdominal pain and diarrhoea, which starts as non-bloody and becomes bloody (Nataro and Kaper 1998). A proportion of cases do not show bloody diarrhoea, but this is difficult to assess accurately as many of these cases are not sampled; 10 % (Nataro and Kaper 1998) and 26 % (Ryan et al. 1986) of cases have been quoted. Vomiting and fever may be present. HUS occurs predominantly in children, with an incidence of 10 % in EHEC patients less than 10 years old. It proves fatal in 3 to 5 % of affected children, with severe neurological and/or renal sequelae in a further 12 to 30 % (Nataro and Kaper 1998).

1.4.2 Prototype EHEC: E. coli O157:H7

1.4.2.1 Lineage and evolution

EHEC O157:H7 shows a close association between serotype and pathotype. *E. coli* O157:H7 is not closely related to a reference EPEC strain, other STEC, other non-EHEC *E. coli* O157 serotypes, or other *E. coli* associated with bloody diarrhoea (Whittam et al. 1993; Perna et al. 1998). Phylogenetic studies on metabolic genes support a recent common ancestor for *E. coli* O157:H7 and EPEC O55:H7, with *E. coli* O157:H7 acquiring Shiga toxin genes and *E. coli* O55:H7 acquiring EAF (Kaper 1998). Studies of archival clinical *E. coli* isolates have revealed that *E. coli* O157:H7 was a rare serotype before 1983 (Nataro and Kaper 1998). The chromosome of *E. coli* O157:H7 is, at 5.5 Mbp, 859 kbp larger than that of the reference *E. coli* K12 laboratory strain (Hayashi et al. 2001). Much of the additional DNA appears to be the result of horizontal transfer into the genome, although many putative genes span junctions between native and inserted sequences (Perna et al. 2001).

1.4.2.2 Virulence factors

Due to the severity of the induced disease, human volunteer studies are not possible for evaluation of virulence attributes. Therefore the identification and evaluation of putative virulence factors relies on epidemiological observation, animal models and *in vitro* studies. The subject has been reviewed by Nataro and Kaper (1998).

A. Shiga toxins

Shiga toxin is believed to be of primary importance in the pathology of HC and HUS, and HUS is most often associated with Stx2. *E. coli* O157:H7 usually produces Stx2 alone or Stx1 plus Stx2 (Griffin and Tauxe 1991), at moderate or high levels (Marques et al. 1986). The status of Shiga toxin-converting prophages in *E. coli* O157:H7 is variable, i.e. one of two in strain EDL933 appears capable of lytic growth (Perna et al. 2001), whereas both in strain O157 Sakai appear defective (Hayashi et al. 2001). This indicates likely variability in expression of the toxins between strains. The RecA protease has a role in de-repressing phage induction in lysogenised bacteria, and in EHEC O157:H7 strains EDL933 and 86-24 it is associated with a high level of Stx2 production and induction of Stx2 by the DNA alkylating agent mitomycin C (Fuchs et al. 1999). This indicates that in those strains, Stx2 production is closely associated with the lytic cycle of the toxin-converting bacteriophage. Recently Shiga toxin-negative *E. coli* O157:H7 and *E. coli* O157:H- strains have been isolated, in the absence of any other STEC, from patients with HUS (Schmidt et al. 1999). The significance of this finding is unknown.

B. Attaching-effacing phenotype

E. coli O157:H7 contains the LEE and expresses *eaeA*-dependent AE lesions *in vitro* and in pig, rabbit, calf and chicken models (Section 1.4.2.7). AE lesions have not yet been demonstrated in natural human disease (Nataro and Kaper 1998), which may reflect late or infrequent sampling of clinical cases. The *E. coli* O157:H7 LEE, at 43.4 kbp, is larger than the LEE of EPEC O127:H6 (Elliott et al. 1998) and is necessary for AE lesions (Donnenberg et al. 1993b; Dean-Nystrom et al. 1998). However, in contrast with the EPEC LEE, it is not sufficient on its own to induce FAS in the HEp-2 assay or to confer the secretion of Esp proteins upon a K-12 laboratory strain into which it was cloned (Elliott et al. 1999). Clear differences between EPEC and *E. coli* O157:H7 are emerging (Section 1.2.3), specifically in respect of quorum-sensing regulation systems involving the LEE (Section 1.2.3.1) and of AE lesion mechanisms (Ismaili et al. 1998). One effect of these differences is that signal transduction in the EHEC O157:H7 AE lesion occurs more slowly than in EPEC-induced AE lesions (DeVinney et al. 1999). There appears also to be a role for the pO157 virulence plasmid in the normal expression of the *E. coli* O157:H7 AE phenotype (Section 1.4.2.2E).

C. Chemical resistance

Pathogenic and non-pathogenic isolates of *E. coli* typically demonstrate a high degree of acid tolerance (Lin et al. 1996; Small 1998), frequently showing greater than 10 % survival in a simulated fasted gastric environment of pH 2.0 for 2 hours (Gorden and Small 1993). *E. coli* O157:H7 is not exceptional within this group (Benjamin and Datta 1995; Lin et al. 1996; Duffy et al. 2000). Prior exposure to acid enhances (Benjamin and Datta 1995) but is

not necessary (Small 1998) for acid tolerance in *E. coli*. Acid tolerance is maximal in late stationary growth phase, when *E. coli* cells are physiologically adapted to environmentally stressful conditions (Benjamin and Datta 1995). The *rpoS* gene, encoding the RpoS stationary-phase RNA polymerase sigma factor (Lange and Hengge-Aronis 1991) is associated with acid, heat and salt tolerance in *E. coli* O157:H7 (Cheville et al. 1996). Several mechanisms for acid tolerance have been proposed, including acid-induced amino acid decarboxylases and an acid-induced oxidative system (Lin et al. 1996; Small 1998). Whilst most *E. coli* O157:H7 strains isolated from humans are resistant to inorganic acid, a small proportion are acid-sensitive with an associated *rpoS* lesion (Waterman and Small 1996), suggesting that normal Rpos expression and/or acid tolerance may not be an absolute prerequisite for virulence. Weak acids, including volatile fatty acids (VFA) found at substantial concentrations in the rumen and intestine, are potent antibacterial agents at low pH, possibly due to their ability to penetrate into the bacterial cytoplasm in the undissociated state (Benjamin and Datta 1995; Booth et al. 1999). The relative contributions of the proton and anion components of the intracellular VFA to bacterial cell damage remains a matter of debate (Russell 1992). The induced acid tolerance of *E. coli* has been shown to have a protective effect against VFA, evident even under mildly acidic conditions (Benjamin and Datta 1995; Lin et al. 1996).

The induced acid tolerance mechanisms have been shown to persist for at least 28 days in cold storage at 4 °C (Lin et al. 1996). This raises the possibility that *E. coli* O157:H7 cells which are acid-tolerised in the intestinal tracts of reservoir hosts or during acidic food preservation processes may enter the human stomach already demonstrating enhanced acid resistance. *E. coli* O157:H7 has been shown to survive for weeks in acidic foodstuffs such as salami, apple juice and cheese (Meng and Doyle 1998). *E. coli* O157:H7 is not exceptionally salt tolerant (Meng and Doyle 1998), but the low infectious dose means that salt-preserved foods may still pose a risk of transmission.

D. Non-intimate adhesion

The initial adhesion of *E. coli* O157:H7 to the intestinal epithelium is poorly understood. *In vitro* adhesion assays produce conflicting results, possibly due to differing methodologies and bacterial strains. No association has been shown between the possession of pO157 and adherence to rabbit ileum *in vitro* (Ashkenazi et al. 1992) or to pig large intestine *in vivo* (Tzipori et al. 1987; Tzipori et al. 1989), but the pO157 plasmid has been shown to confer a colonisation advantage in streptomycin-treated mice (Wadolkowski et al. 1990). One study suggested that pO157 conferred fimbriae, and adhesion to Henle (Int) 407 cells (Karch et al. 1987), but other studies failed to confirm this (Clausen and Christie 1982; Toth et al. 1990). There is a report of a homologue of the *irgA*-encoded protein of *Vibrio cholerae*, named Iha,

There is a report of a homologue of the *irgA*-encoded protein of *Vibrio cholerae*, named Iha, associated with adherence of *E. coli* O157:H7 to HeLa cells (Tarr et al. 2000). Deletion mutants of *E. coli* O157:H7 lacking *eaeA* do not adhere, or adhere in much smaller numbers, to HEp-2 and HeLa cells than do their wild-type counterparts (McKee et al. 1995a; DeVinney et al. 1999). The residual adhesion is diffuse rather than microcolonial. These effects have also been demonstrated with a Tir-deficient mutant on HeLa cells (DeVinney et al. 1999) and with various LEE mutants on Caco-2 cells (Tatsuno et al. 2000). By contrast, intimin-deficient mutants of an EAF-positive EPEC retained their ability to form microcolonies on HEp-2 cells (Jerse et al. 1990). Adhesion of *E. coli* O157:H7 to HEp-2 cells is low or absent after three hours' incubation (Karch et al. 1987; Tesh and O'Brien 1992), but reliably present after five or six (Sherman et al. 1987; Tesh and O'Brien 1992; Donnenberg and Nataro 1995; McKee and O'Brien 1995b).

Type-I fimbriae have been shown to be associated with adherence of *E. coli* O157:H7 to human epithelium and to rabbit ileal brush border *in vitro* (Durno et al. 1989), however most *E. coli* O157:H7 strains contain a 16 base-pair deletion in the fim switch controlling the *fimA* gene, abolishing expression of type-1 fimbriae (Enami et al. 1999; Iida et al. 2001; Roe et al. 2001). Analysis of the O157 Sakai strain of *E. coli* O157:H7 has revealed 14 sets of genes encoding putative fimbrial biosynthesis systems (Hayashi et al. 2001), four of which have only been found in *E. coli* O157:H7. However, the expression and significance of these sequences are undetermined. Expression of curli fibres by *E. coli* O157:H7 is uncommon and unstable, but has been reported in some human-derived strains, associated with variations in the *csgDEFG* operon (Uhlich et al. 2001). There is *in vitro* evidence of an adhesive effect due to OMP (Sherman et al. 1987; Sherman and Soni 1988b; Sherman et al. 1991), particularly a 94 kDa OMP which is not intimin (Louie et al. 1993) and which is not associated with pO157 (Dytoc et al. 1993), and an 8 kDa OMP which also showed adhesive qualities *in vivo*, in chicken caeca (Zhao et al. 1996). Other proposed adhesins borne by *E. coli* O157:H7 are lipopolysaccharide (Paton et al. 1998) and exopolysaccharide (Junkins and Doyle 1992). Furthermore, *E. coli* O157:H7 and EPEC appear to adhere, by an unknown mechanism, to phosphatidylethanolamine, a surface-exposed phospholipid of the eukaryotic cell membrane (Barnett Foster et al. 1999; 2000). There is evidence for an adhesive role for EspA filaments (Section 1.2.3.2A), but whether these structures have a primary role in initial adherence to mucosal surfaces *in vivo* is uncertain.

In summary, there are reports of fimbrial and of non-fimbrial non-intimate adhesins and receptors. However, the significance of these putative adhesive mechanisms *in vivo* is undetermined. The marked reduction in adhesion to cell monolayers *in vitro* of intimin and Tir mutants of *E. coli* O157:H7 suggests two possibilities. These are that initial adhesion may be transient and the formation of AE lesions is necessary for persistent adhesion, and/or

that these LEE-encoded molecules play a broader role, mediating non-intimate adhesion in addition to their established interaction in the AE lesion.

E. Other putative virulence determinants

The pO157 plasmid is present in virtually all *E. coli* O157:H7 isolates (Levine et al. 1987) and in many other human-derived STEC where its presence, indicated by detection of enterohaemolysin, is associated with the development of HUS (Schmidt and Karch 1996). Several putative virulence factors are encoded on the pO157 plasmid (Burland et al. 1998), as follows. Enterohaemolysin, encoded by the EHEC-*hlyABCD* operon, is believed to release iron for bacterial growth from host cells by lysis. Two other haemolysins, Ehly1 and Ehly2, are encoded on pO157 and are widespread amongst STEC, but their significance is unknown. The plasmid contains a 13-gene cluster, termed *etpC* to *etpO*, homologous with genes encoding a general (type II) protein secretory system in other Gram-negative bacteria (Schmidt et al. 1997). A probe for *etpD* revealed that it is highly prevalent in EHEC O157:H7 (100 % of those tested), sporadic in non-O157 EHEC (60 %) and uncommon in bovine STEC (10 %). It is not known whether the *etp* genes are functional. Catalase P (encoded by *katP*) is a catalase-peroxidase, which is thought to protect against oxidative stress, as induced during phagocytosis by host cells (Brunder et al. 1996; Burland et al. 1998). An iron-transport OMP is present which may bind iron for bacterial use in low-iron environments. EspP, a serine protease, may be associated with increased haemorrhage (Burland et al. 1998). Sequencing of pO157 has revealed possible genes for fimbriae, *per*-like regulator sequences (Burland et al. 1998), and a gene (*toxB)* which appears to have a positive, probably post-transcriptional, role in the level of expression of some LEE proteins, and in the expression of the normal adherence phenotype *in vitro* (Tatsuno et al. 2001). Finally, pO157 has been reported to promote the transcription of the *esp* operon of the LEE (Beltrametti et al. 1999). These last two reports suggest a role for a product or products of the plasmid in promoting the formation of the AE lesion.

Concerning features not associated with pO157, encapsulated isolates have been reported from an outbreak (O'Brien et al. 1993), and appear to be common, even typical (Junkins and Doyle 1992) but their significance has not been further discussed. Entero-aggregative *E. coli* stable toxin 1 (EAST-1), which has homology to the ETEC stable toxin, is encoded on the *E. coli* O157:H7 chromosome and is of unknown significance, but may be important in cases of non-bloody diarrhoea (Nataro and Kaper 1998). Alpha-haemolysin may be present but it is not associated with increased virulence.

1.4.2.3 Enteric pathology in humans

The lack of AE lesions observed in human biopsy and necropsy material may be due to sampling late in the disease and/or infrequently (Nataro and Kaper 1998). Colonoscopy and biopsy reports from cases of HC reveal a multifocal pattern of damage, and two histopathological patterns have been described. One ('ischaemic') suggests an underlying vasculopathy and shows mucosal necrosis, plus or minus ulceration, with capillary thrombosis and little inflammation (Brownlie and Grau 1967; Griffin et al. 1990; Kelly et al. 1990). The other ('infectious') shows an acute inflammatory picture with cryptitis and crypt abscessation (Griffin et al. 1990). The two patterns may coexist in an individual.

1.4.2.4 Epidemiology in human infection

In North America, *E. coli* O157:H7 causes 15 to 39 % of bloody diarrhoea cases and is the most frequent pathogen isolated from bloody stools (Pai et al. 1984; Remis et al. 1984). The incidence is seasonal, with a maximum between June and September in the Northern Hemisphere (Boyce et al. 1995; Anon. 2002). Identified sources include beef (particularly hamburgers), other meats including sheep meat (Chapman et al. 2001) and preserved meats such as salami, water, milk including sheep milk (Rubini et al. 1999), apple juice and raw vegetables (Griffin and Tauxe 1991; Nataro and Kaper 1998). Contact with animal faeces was a strong risk factor for sporadic *E. coli* O157:H7 infection (Locking et al. 2001), and traceable links between human infection and ruminant faeces have been made (Licence et al. 2001; Strachan et al. 2001). Interpersonal spread is frequent, accounting for approximately 20 % of cases, and consequently a biphasic pattern of disease in outbreaks is often seen (Ryan et al. 1986; Cryan 1990). Both outbreaks and sporadic cases occur (Riley et al. 1983; Pai et al. 1984; Remis et al. 1984). The duration of excretion is most prolonged in children, but does not exceed a few weeks, and long-term asymptomatic carriage is believed to be rare or non-existent (Boyce et al. 1995).

The causal link between *E. coli* O157:H7 and HC/HUS is postulated because of the association of the serotype with the diseases, the absence of the serotype in unaffected individuals, and the consistent presence in isolates of the virulence attributes of Shiga toxin and AE phenotype. The low prevalence of *E. coli* O157:H7 in food samples compared with other STEC implies a high pathogenic potential (Johnson et al. 1996). Investigation of sources in outbreaks indicates that the infective dose is very low, of the order of 100-200 organisms (Nataro and Kaper 1998) or even lower (Meng and Doyle 1998; Strachan et al. 2001), and it is believed that the infective dose may be below the limit of detection in foodstuffs (Tarr and Neill 1996).

There is a marked geographical variation in incidence even within the developed world (Cryan 1990), and there is growing evidence that *E. coli* O157:H7 is prevalent within parts of the developing world (Cunin et al. 1999; Gwavava et al. 2001).

1.4.2.5 Detection in human, veterinary and food samples

Because of the low infective dose and the presence of commensal *E. coli* in patients and animals, the reliable detection of *E. coli* O157:H7 from food, faeces and carcasses requires methods with high sensitivity and specificity. *E. coli* O157:H7 does not ferment sorbitol rapidly, but 80 to 90 % of commensal *E. coli* do (Boyce et al. 1995), and this permits sorbitol-McConkey agar (SMAC) to be used as an indicator medium. Incorporation of rhamnose, which *E. coli* O157:H7 does not ferment, assists discrimination between O157:H7 and other sorbitol-negative *E. coli*. There is an association between *E. coli* O157:H7 and a plasmid-borne gene associated with tellurite resistance, which permits the selective inhibition of non-O157:H7 *E. coli* on tellurite agar (Zadik et al. 1993). Sorbitol-MacConkey agar supplemented with cefixime and tellurite has proved to be a reliable selective medium for the detection of *E. coli* O157:H7 (Chapman 2000a). Another distinguishing biochemical feature of the bacterium is the lack of β-glucuronidase, which ferments 4-methyl umbelliferyl-β-D-glucuronide (MUG) to a fluorescent product. Therefore, in contrast to most *E. coli*, *E. coli* O157:H7 is MUG-negative on fluorescent examination of appropriate media. A number of commercial selective solid media are available for the culture of *E. coli* O157:H7, some of which have chromogenic or fluorogenic indicator properties. The various media available differ in sensitivity and specificity (Manafi and Kremsmaier 2001), and detection protocols involving subculture on more than one medium appear to improve detection rates (Wallace and Jones 1996; Wallace et al. 1997).

For detection by culture, enrichment of samples in broths containing antibiotics or other differentially suppressive agents, with or without subsequent immunomagnetic separation (IMS) using magnetic beads coated with O157 antibodies, increases sensitivity. In one study, enrichment in buffered peptone water (BPW) with vancomycin, cefixime and cefsulodin followed by IMS and culture on CT-SMAC agar was shown to be approximately 100 times more sensitive than direct culture on CT-SMAC for the detection of *E. coli* O157:H7 in bovine faeces (Chapman et al. 1994). A similar advantage in sensitivity for IMS over enrichment plus direct subculture was demonstrated for minced beef samples in another study (Wright et al. 1994) but with poor repeatability at the lowest detectable concentration of 2 cfu/g. Using enrichment protocols, in one report IMS proved to be some 100 times less sensitive in the detection of O157 organisms from spiked bovine faeces (requiring around 100 cfu/g for reliable detection) than from similarly spiked food or milk samples (Illingworth

1998). Similarly, another report (Eriksson et al. 1998) showed, using a similar protocol to that in the present studies (pre-enrichment in BPW for six hours, IMS and culture on CT-SMAC), that the sensitivity of IMS fell below 100 % when the *E. coli* O157:H7 concentration in spiked bovine faeces was less than 100 cfu/g. Sanderson et al. (1995) inoculated calves orally with *E. coli* O157:H7 and used enrichment of faeces samples in tryptone soya broth plus cefixime and vancomycin (TSBcv) followed by either direct subculture of the diluted broth on CT-SMAC, or IMS plus subculture on CT-SMAC. No significant difference in sensitivity of detection was found between the IMS and non-IMS methods, when the enriched direct subculture method was optimised. With either technique, it was concluded that reliable detection of less than 100 cfu/g bovine faeces was not possible. However a similar comparison in another report (Besser et al. 2001) found IMS to be significantly more sensitive than TSB_{CV} enrichment, with final culture on SMAC in both techniques.

The precise methodology used with any approach to detection appears potentially to substantially affect the eventual sensitivity. For example, when using broth incubation prior either to direct subculture or to IMS, enrichment using antibiotics or other selective agents is commonly practised (Chapman 2000a). However, omitting selective agents at this stage (pre-enrichment) improves the recovery rate of sublethally damaged *E. coli* O157:H7 from environmental or food sources (De Boer and Heuvelink 2000), and non-selective pre-enrichment using BPW alone has proved more sensitive than enrichment using antibiotics, in an IMS protocol using bovine faeces samples (Synge et al. 1998). The type of solid media used for final culture also appears to the affect sensitivity of detection, with one group concluding that CHROMagar O157 was most the sensitive of a range of selective media tested, using bovine and bird faeces samples (Wallace and Jones 1996; Wallace et al. 1997). Some field (Heuvelink et al. 2001) and experimental (Besser et al. 2001) studies suggest that IMS, and particularly pre-enriched IMS (Section 1.4.2.6), is the most sensitive cultural method of detection of *E. coli* O157:H7 from ruminant faeces.

Sensitive non-culture assays for the detection of *E. coli* O157:H7 include polymerase chain reaction (PCR) for various elements with specificity for *E. coli* O157:H7 (Campbell et al. 2001), enzyme-linked immunosorbent assay (ELISA; Kerr et al. 2001), and nucleic acid microarrays (Call et al. 2001). Detection of Shiga toxin or Shiga toxin genes may be attempted from stool or culture, but the genes may be lost from the organism during subculture (Karch et al. 1992). CVD419 is an empirically-derived polynucleotide probe which may be used for the detection of the large EHEC plasmid pO157 (Levine et al. 1987), which was present in 99 % of *E. coli* O157:H7 strains tested. However CVD419, subsequently shown to recognise part of the EHEC-*hly* enterohaemolysin gene cluster (Schmidt et al. 1995), lacks specificity for *E. coli* O157:H7 amongst STEC.

1.4.2.6 Occurrence and epidemiology in veterinary species

Cattle

Very few isolates of *E. coli* O157:H7 were reported from cattle before 1982 (Orskov et al. 1987). The field and experimental reports concerning *E. coli* O157:H7 epidemiology in cattle are summarised in Appendix 4. Due to the concentration of *E. coli* O157:H7 excreted by healthy cattle, often less than 10^3 cfu/g faeces (Sanderson et al. 1995) compared with a typical density of 10^6 *E. coli* per gram of colon contents (Hartl and Dykhuizen 1984), the detection method employed is likely to have had a significant effect on prevalence and persistence data. In published surveys the detection methods vary, but typically involve broth enrichment then subculture on a solid medium, or IMS and culture following pre-enrichment or enrichment. Enrichment broths used include: TSB with vancomycin, cefixime and cefsulodin added in varying combinations to inhibit the growth of Gram positive organisms, *Proteus* and *Aeromonas* respectively (Hancock et al. 1994; Sanderson et al. 1995; Hancock et al. 1997b; Rice et al. 1997; Hancock et al. 1998b; Van Donkersgoed et al. 2001); modified *E. coli* medium (mEC; Garber et al. 1999), mEC plus novobiocin to inhibit Gram positive bacteria (Faith et al. 1996; Dargatz et al. 1997; Shere et al. 1998); TSB with antibiotic(s) plus bile salts (Cray and Moon 1995; Garber et al. 1995; Brown et al. 1997; Harmon et al. 1999; Cornick et al. 2002) or tellurite (Hovde et al. 1999; Magnuson et al. 2000) or both (Cray et al. 1998) as additional selective agents. Enriched mixtures typically have been subcultured onto CT-SMAC. IMS has been performed on mixtures pre-enriched in Tris buffered saline (TBS; Buchko et al. 2000a; Lahti et al. 2001) or BPW (Nielsen 2001; Synge et al. 2001), or following enrichment in antibiotic-supplemented BPW, TSB, or mEC (Sanderson et al. 1995; Besser et al. 1997; Chapman et al. 1997; Heuvelink et al. 1998a; Richards et al. 1998; Akiba et al. 2000; Wray et al. 2000; Chapman et al. 2001; Johnsen et al. 2001).

The reported prevalence of *E. coli* O157:H7 excretion by cattle varies between 0 % (Hancock et al. 1997a; Mechie et al. 1997; Shere et al. 1998) and 68 % of individuals (Mechie et al. 1997). Part of this apparent variability may be attributed to temporal variation in excretion (Hancock et al. 1997b), with peak prevalences generally seen during the summer months (Chapman et al. 1997; Mechie et al. 1997; Heuvelink et al. 1998b; Conedera et al. 2001). However, as sampling and detection protocols vary widely (Appendix 4), it is difficult to gauge the extent to which a reported difference in prevalence reflects a genuine difference in the excretion pattern. The data from Appendix 4 shows that most reported prevalences of *E. coli* O157:H7 excretion fall within the range 0.5 to 5 % for adults and calves. Where IMS is employed, values are often higher, for example extensive surveys on farms in Scotland (Synge et al. 2001) and at an abattoir in England (Chapman et al. 2001)

yielded prevalence rates of 7.9 % and 12.9 % respectively. Geographical variation in prevalence may play a role in such observed differences. The reported prevalence of *E. coli* O157:H7-excreting herds is 10 to 75 %.

Where both calves and older stock have been sampled in the same study, weaned calves have generally been found to exhibit a higher prevalence of *E. coli* O157:H7 excretion (Heuvelink et al. 1998b; Richards et al. 1998; Cobbold and Desmarchelier 2000). Prevalences of 2.8 and 0.15 % for calves and adults respectively were determined in one case (Wells et al. 1991). The prevalence of excretion of *E. coli* O157:H7 increases at the weaning period (Garber et al. 1995), and group housing of calves appears to increase the risk of excretion (Garber et al. 1995; Shere et al. 1998). The duration of excretion of the organism also appears to be more extended in calves when compared with adults (Mechie et al. 1997). Identified risk factors for *E. coli* O157:H7 excretion include: weaning, group housing of preweaned calves, poor calf feeding hygiene, dietary changes and transport to slaughterhouse lairage (Suthienkul et al. 1990; Garber et al. 1995; Mechie et al. 1997). Increased excretion of the organism following dietary changes is supported by studies showing that starvation and irregular feeding greatly increases the *E. coli* density in the rumen and large intestine of cattle and sheep (Brownlie and Grau 1967; Rasmussen et al. 1993). There is no association with illness; indeed there was a negative association between diarrhoea and the presence of *E. coli* O157:H7 in faeces samples received at a diagnostic laboratory (Richards et al. 1998).

There is no clear evidence of a consistent difference in the prevalence of excretion of *E. coli* O157:H7 between different types of cattle farming enterprise, although relatively few comparative studies have been performed. In one such study, the prevalences of excretion from dairy, feedlot and extensive beef farms were 0.33, 0.28 and 0.71 % respectively (Hancock et al. 1994).

Longitudinal surveys show that, whilst a herd's *E. coli* O157:H7-positive status tends to be maintained over time (Hancock et al. 1997a; Hancock et al. 1997b; Nielsen 2001), excretion of the organism by individuals typically is of short duration, with only a minority of animals found to be excreting on consecutive sampling occasions (Mechie et al. 1997; Heuvelink et al. 1998b; Shere et al. 1998; Conedera et al. 2001). Intermittent excretion is often seen (Mechie et al. 1997; Conedera et al. 2001) which, it may be hypothesised, in any particular instance might arise from: repeated recolonisation of an individual, persistent colonisation with bouts of excretion, or continuous excretion around the lower limit of detection. In many longitudinal surveys there are animals which appear to excrete continuously for prolonged periods, ranging from four weeks (Mechie et al. 1997) to eight months (Conedera et al. 2001). Although the temporal resolution of these studies is limited by sampling intervals typically of one month, but up to three months in some cases, it appears that within herds there may be a few persistently-excreting individuals which can

help to maintain *E. coli* O157:H7 within the group as a whole. In experimental oral inoculation studies with frequent sampling intervals and fewer animals, continuous excretion has been seen for 27 weeks in calves (Cray and Moon 1995), 69 days in heifers (Magnuson et al. 2000), and 67 days in yearlings (Buchko et al. 2000b). Inter-animal variation in the tendency to excrete persistently has been observed experimentally (Grauke et al. 2002).

Many longitudinal surveys over periods of 11 to 15 months have shown that *E. coli* O157:H7 tends to persist within a herd (Besser et al. 1997; Hancock et al. 1997a; Mechie et al. 1997; Hancock et al. 1998b; Sargeant et al. 2000; Conedera et al. 2001; Galland et al. 2001). One study has suggested additionally that *E. coli* O157:H7-positive herds may have an inherent and stable 'high' or 'low' prevalence (Hancock et al. 1997b). Despite this, the prevalence within excreting herds is seen to vary, sometimes widely, between sampling occasions (Hancock et al. 1997a; Hancock et al. 1997b; Mechie et al. 1997; Heuvelink et al. 1998b; Conedera et al. 2001). Herds which have a zero prevalence of *E. coli* O157:H7 excretion at the beginning of a study have subsequently been observed to convert to 'excreting' herds (Heuvelink et al. 1998b).

E. coli O157:H7 strains present on farm environments have been investigated, principally using pulsed-field gel electrophoresis (PFGE) to delineate strains and strain variation over time. Some PFGE types appear to be endemic, i.e. persistent, on any one premises, whether dairy (Shere et al. 1998; Nielsen 2001) or feedlot (Van Donkersgoed et al. 2001). Other strains present are more transient. Identical or very similar PFGE types may show a wide geographical distribution in the USA (Rice et al. 1999). The number of PFGE strain types present on a premises at any one time may be as many as seven, and does not appear to correlate with the degree of animal movements onto the farm (Rice et al. 1999). It is uncertain to what extent the PFGE types present on a farm are from different sources, as calves experimentally inoculated with *E. coli* O157:H7 showed alterations in the PFGE profile of recovered *E. coli* O157:H7 over 63 days (Akiba et al. 2000).

Where evidence of *E. coli* O157:H7 in the farm environment has been sought, water sources (Faith et al. 1996; Hancock et al. 1998b; Sargeant et al. 2000; Conedera et al. 2001; Van Donkersgoed et al. 2001) and feed bunks (Van Donkersgoed et al. 2001) have proved positive in a small proportion of cases. Water contamination by *E. coli* O157:H7 (Shere et al. 1998) and the use of water troughs rather than individual drinkers by dairy cattle (Garber et al. 1999) were both shown to be risk factors for excretion of the organism. Furthermore, in one study PFGE typing has shown that strains from cattle and their environment were of the same PFGE type (Hancock et al. 1998b). Oral swabs from yearlings in an *E. coli* O157:H7-positive group yielded the organism in 30 of 180 cases (Buchko et al. 2000b). Extensive surveys have identified housing as a risk factor for the excretion of *E. coli* O157:H7 by adult cattle (Synge et al. 2001), and a six-month study demonstrated a markedly higher prevalence

of *E. coli* O157:H7 excretion in a group of indoor-housed calves than in a group at pasture (Jonsson et al. 2001). The above evidence strongly suggests a significant role for the farm and lairage environment in the maintenance of *E. coli* O157:H7 excretion by a herd, probably by frequent re-ingestion of the organism. Indeed, the assumed 'reservoir' status of cattle has been questioned (Hancock et al. 1998b), it being suggested that persistence on a farm may be primarily environmental and that cattle are not uniquely or particularly susceptible to persistent colonisation. In this scenario cattle, and perhaps other animals, may act as transiently-infected 'multipliers' for an environmentally-persistent reservoir of *E. coli* O157:H7.

E. coli O157:H7 has been recovered from milk samples in one study but not in another (Wells et al. 1991; Mechie et al. 1997), and the screening of bulk milk tank samples and slurry has not been found to be sensitive for detection of *E. coli* O157:H7 positive herds (Garber et al. 1995). Correlations have been noted between the seasonal incidence of herd excretion and human disease (Mechie et al. 1997) and between excretion on particular farms and human EHEC O157:H7 cases (Wells et al. 1991).

In summary, there is epidemiological evidence for persistent colonisation by *E. coli* O157:H7 of cattle herds, and of more transient groups such as in feedlots. However, at the individual animal level, persistent excretion is observed in only a small minority of animals. Weaned calves show the greatest prevalence and duration of excretion of the organism, and dietary changes and stress appear to promote excretion. The farm environment, and particularly the water supply, appears to harbour the organism, and environmental niches outside the animal are likely factors in the maintenance of *E. coli* O157:H7 on a premises. Certain strains of the organism appear to persist on a farm for longer than others, either by virtue of adaptation or ubiquity, although several strains may co-exist on premises at any one time. Excretion may be higher at the slaughterhouse than on the farm. There is no evidence that *E. coli* O157:H7 is pathogenic in naturally exposed cattle.

Other ruminants

E. coli O157:H7 has been reported in ovine faeces from a number of studies in the UK and USA. Prevalence values are subject to the same influences of sampling protocols and detection methods as are those of cattle. The range, from surveys sampling between 325 and 7200 animals, is 0 to 7.4 % of animals excreting *E. coli* O157:H7 (Chapman et al. 1996; Chapman et al. 1997; Kudva et al. 1997b; McCluskey et al. 1999; Chapman et al. 2001; Heuvelink et al. 2001; Johnsen et al. 2001; Paiba et al. 2002). Where prevalence surveys of cattle and sheep *E. coli* O157:H7 excretion have been conducted contemporaneously, values for sheep have been lower than for cattle (Chapman et al. 1997; Chapman et al. 2001; Paiba et al. 2002). A summer/autumn peak in the prevalence of excretion has been found (Kudva et

al. 1996; Heuvelink et al. 2001; Paiba et al. 2002). The proportion of animals in a group excreting the organism appears to increase with stressful situations, such as long transport or prolonged detainment in holding pens (McCluskey et al. 1999). Faecal isolates typically possess the normal EHEC O157:H7 virulence attributes (Heuvelink et al. 2001).

E. coli O157:H7 has been detected in wild deer faeces (Fischer et al. 2001) and in venison (Zhao et al. 1998).

Pigs

There is evidence that *E. coli* O157:H7 with typical EHEC characteristics is common in South American domestic pigs (Borie et al. 1997; Notario et al. 2000), and the organism has been found in European pig herds (Heuvelink et al. 1999; Johnsen et al. 2001).

Other species

E. coli O157:H7 has been isolated from many non-ruminant animals, including flies (Hancock et al. 1998; Heuvelink et al. 1998b; Kobayashi et al. 1999), equids (Hancock et al. 1998b; Heuvelink et al. 1998b; Bauwens et al. 2000; Chapman et al. 2000a) and rabbits (Pritchard et al. 2001); in the last case there was evidence of a vector role in human disease.

1.4.2.7 Experimental studies on *E. coli* O157:H7 in animals

Introduction

Due to the generally low pathogenicity of *E. coli* O157:H7 in non-human species, animal models of human disease generally require very high oral doses, in the order of 10^6 to 10^{10} colony-forming units (cfu), in comparison with the human infective dose, plus other features such as colostrum deprivation, suppression of the normal intestinal flora, or gnotobiotic status.

Cattle

Studies using Shiga toxin-producing and *eaeA*-positive *E. coli* O157:H7 strains have shown large intestinal AE lesions plus colonic oedema and inflammation in colostrum-deprived (CD) calves inoculated orally at less than 12 hours of age, with less marked changes in calves inoculated at three days of age (Dean-Nystrom et al. 1997). This age-related resistance has been supported by studies using five-day old gnotobiotic calves (Woodward et al. 1999), three- to 14-week old preweaned calves (Cray and Moon 1995), and eight-week old weaned calves (Brown et al. 1997), in which no AE lesions were seen. Some mild intestinal inflammation and haemorrhage was seen in the five-day old calves (Woodward et al. 1999). Transient non-bloody diarrhoea was seen in several studies even in calves without detectable AE lesions. One study of neonatal CD calves reported *eaeA*-

dependent blood-tinged diarrhoea, AE lesions, intestinal oedema and haemorrhage, villus atrophy and fibrinous mucosal exudation (Dean-Nystrom et al. 1998). The susceptibility of three- to four-month old weaned calves to *E. coli* O157:H7-induced AE lesions in the large intestine has been demonstrated (Dean-Nystrom et al. 1999), but only in those animals where a luminal concentration of the organism in excess of $10^{5.5}$ cfu/g was achieved, via fasting prior to inoculation and sampling within a few days of inoculation.

Examination of persistence characteristics has revealed that calves experimentally inoculated with 10^{10} cfu of *E. coli* O157:H7 excreted the organism for longer and in greater quantities than did adult cattle (Cray and Moon 1995). One calf in this study excreted consistently for at least 27 weeks. *E. coli* O157:H7 was consistently isolated from the rumen and large intestine, with additional isolation from the small intestine and gut-associated lymphoid tissue in some calves. In the same report, oral doses of 10^{7} and 10^{4} cfu *E. coli* O157:H7 in adults established excretion of *E. coli* O157:H7 in only two of 10 animals, compared with all 12 adults inoculated with 10^{10} cfu. That the rumen and large intestine are sites of persistence in calves was confirmed by another study of isolated calves inoculated with 10^{10} cfu *E. coli* O157:H7 which excreted the organism for at least 20 days and in which consistent recovery of the organism was obtained from the rumen, with frequent recovery from the large intestine (Brown et al. 1997). A calf study using an oral dose of 5×10^{8} cfu showed excretion times, of about 1 month, preweaning (Sanderson et al. 1999). Re-inoculation at 12 and 18 weeks produced reduced excretion times of about 1 week. Whether this was due to the altered intestinal flora of the weaned calf or to prior exposure was not investigated. Despite the apparent correlation between dose and excretion reported by Cray and Moon (1995), Besser et al. (2001) reported that some, but not all, bovine-derived strains of *E. coli* O157:H7 would establish persistent excretion from some calves, and would transmit between calves, following oral doses as low as 210 cfu. Histopathological examination of the intestinal tissues of persistently-excreting calves has not yielded proof of AE lesion formation (Cray and Moon 1995; Brown et al. 1997; Harmon et al. 1999). Studies using eight-week to 12-month old calves (Naylor et al. 2003), reported after completion of the work contained in this thesis, have shown that the terminal rectum and anus is a preferential site of colonisation by *E. coli* O157:H7 in persistently-excreting animals. O157-bearing bacteria formed microcolonies at this site in both experimentally and naturally excreting calves, but the nature of the attachment was not determined.

Studies on the effect of starvation upon the density of *E. coli* O157:H7 excreted by weaned calves following oral inoculation have shown a positive effect only when starvation was applied at the time of inoculation (Cray et al. 1998; Harmon et al. 1999). The effects of dietary composition on the excretion of *E. coli* O157:H7 by orally inoculated animals have been the subject of several reports. In one study (Hovde et al. 1999), yearling steers excreted

the organism for longer on a forage-based diet than on a grain-based diet, but two other studies using heifers (Magnuson et al. 2000) and calves (Tkalcic et al. 2000) found no such difference between grain- and forage-based diets.

Two studies have examined the excretion of *E. coli* O157:H7 by dually-inoculated animals. In one, a mutant lacking a functional RpoS global stress response regulator was excreted from calves at a significantly lower level than its wild-type parent strain (Price et al. 2000), although there was no evidence of reduced persistence in the former strain. In the other, an intimin-deficient mutant was excreted at a lower level from yearling cattle than was its wild-type parent strain (Cornick et al. 2002).

A study on the clonal variation over time of an inoculated *E. coli* O157:H7 strain in calves (Akiba et al. 2000) has shown changes in PFGE profile and loss of the pO157 plasmid over a period of 63 days.

In summary, age-related *eaeA*-dependent diarrhoea associated with AE lesions in the distal ileum and the large intestine has been observed, and the capacity of *E. coli* O157:H7 to form AE lesions acutely in calves up to four months old has been demonstrated. Persistence has been demonstrated in the rumen and large intestine of calves. Factors involved in excretion times include dose and either or both of age and prior exposure, although dose-dependence does not appear to apply for all strains and hosts. A likely predilection site for persistence has been identified at the terminal rectum. There is evidence that the intestinal state in respect of starvation and diet does affect excretion, but the relationship is not straightforward. Bacterial factors, including stress responses and intimin may provide a competitive advantage in the intestine. Persisting strains show readily detectable genetic changes over weeks.

Sheep and other ruminants

Experimental studies involving sheep have used similar techniques and oral dosages to those of cattle. Fewer studies have been reported for sheep than for cattle. Findings in respect of dosage-excretion relationships, the persistence of excretion and the significance of intimin are similar to those in cattle.

One study (Kudva et al. 1995) examined the effects of inoculum dose on excretion by 14-day old suckling lambs and two adult rams. A human-derived *E. coli* O157:H7 was inoculated at 10^5 or 10^9 cfu to co-housed animals. Those lambs given the lower dose started excreting the strain later and continued for a shorter period compared with those given the higher dose, although detection was markedly variable amongst both groups. A similar dose effect upon excretion was seen with two adult rams, with the high-dose individual excreting consistently for 50 days post inoculation (p.i.).

The effects of dietary manipulation and repeated inoculation with *E. coli* O157:H7 on faecal excretion of *E. coli* O157:H7 were also examined by the same group (Kudva et al. 1997b). Using a 10^{10} cfu inoculum of either nalidixic acid sensitive or resistant organisms (the latter of bovine origin), excretion was detected for the 41 days of the experiment, although the proportion of excreting individuals declined over time. Differences in the duration of excretion were not observed between the two strains used, but the feeding of a concentrate-based diet appeared to reduce excretion times in comparison with a fibre-based diet. An effect of previous inoculation upon the duration of excretion was not observed. Of five sheep which excreted *E. coli* O157:H7 consistently for 41 days in this study, two did not yield the organism from any intestinal location when examined *post mortem* at 53 days p.i. (Grauke et al. 2002). From the three remaining sheep, samples of faeces (whether these were voided faeces or rectal contents is unspecified) yielded the organism in all three cases, and caecal samples were positive in one case. No other (unspecified) gastrointestinal tract tissues or contents were culture positive for *E. coli* O157:H7, and bacterial lesions were not observed in any (unspecified) tissues examined by microscopy.

Subsequent work by the same group included the oral inoculation of 24 sheep, which were six- to seven-months old and group-housed, with 10^9 cfu of a human-derived strain of *E. coli* O157:H7 (Grauke et al. 2002). At weekly intervals for six weeks, faecal samples were examined from groups of four sheep, which were then examined *post mortem*. A proportion of each group of four were excreting the inoculated organism but, from one week p.i., the inoculated organism was frequently not recovered from the large intestine and rumen of excreting individuals by enrichment culture (TSB_{VCT} then subculture on CVTM-SMAC; see legend, Appendix 4). Indeed, from three to six weeks p.i., only two sites (descending colon and rectum) from one of seven excreting animals proved to be culture positive.

Cornick et al. (2000) examined the persistence of bovine- and human-derived *E. coli* O157:H7 in young adult sheep in comparison with ETEC and EPEC pathotypes of *E. coli*. Doses of 10^{10}, 10^7 and 10^5 cfu, but not 10^4 cfu, produced excretion of *E. coli* O157:H7 for the two months of monitoring. As the initial dose decreased, the proportion of animals excreting at two months p.i. decreased also. Culture of the alimentary tissues of two sheep excreting *E. coli* O157:H7 yielded the organism only in the rectal contents.

The same research group inoculated six- to 12-month old sheep with 10^{10} cfu of an antibiotic resistance marked human-derived *E. coli* O157:H7 strain and its isogenic intimin-deficient mutant, singly and in combination (Cornick et al. 2002). Excretion of both strains was observed up to 60 days p.i. in some animals, although the intimin-deficient mutant appeared to be a poorer coloniser in respect of average duration of excretion. In the same study, yearling cattle provided similar results when dually inoculated.

Another study reported the effects of probiotic feed supplements upon faecal shedding of *E. coli* O157:H7 by 12- to 15-month old sheep following oral inoculation of 10^{10} cfu of the bacteria (Lema et al. 2001). All five groups of six inoculated sheep excreted the *E. coli* O157:H7 consistently, at between 10^{1} and 10^{7} cfu/g faeces, over the seven week period of the study. However, some of the data in this paper relating to animal weights for age, food consumption and feed conversion efficiency appear to be erroneous and the source of the *E. coli* O157:H7 strain given is incorrect, therefore other experimental results from this report might be interpreted with caution.

Fischer et al. (2001) inoculated white-tailed deer with 10^{8} cfu of a mixed *E. coli* O157:H7 inoculum and observed excretion for the 26 days of the study. Mild non-haemorrhagic diarrhoea was seen in a proportion of the animals. The organism was transmitted to an uninoculated in-contact animal by two days p.i. At *post mortem* examination *E. coli* O157:H7 was isolated from the rumen and the small and large intestines, and the deer-derived strain in the mixed inoculum appeared to be the most persistent.

Pigs

Several studies have been performed using *eaeA*- or LEE-positive, Shiga toxin-producing strains and gene deletion mutants in gnotobiotic or CDCD piglets, usually of one to two days of age. AE lesions have been observed in the large intestine plus or minus the distal small intestine (Francis et al. 1986; Tzipori et al. 1986; Tzipori et al. 1987). These lesions have been shown to be *eaeA*-dependent and Shiga toxin-independent (Tzipori et al. 1987; Donnenberg et al. 1993b). Limited mucosal invasion was observed in one study (Tzipori et al. 1986). Associated colonic changes include mesocolonic oedema, gut wall oedema and neutrophil infiltration (Francis et al. 1986). Clinically, non-bloody diarrhoea was seen (Francis et al. 1986), which was independent of Shiga toxin presence (Tzipori et al. 1987). The pO157 plasmid did not appear to affect the AE lesion (Tzipori et al. 1987; Tzipori et al. 1989). Another study has demonstrated the susceptibility of colostrum-fed piglets to AE lesion formation by Shiga toxin-positive and -negative *E. coli* O157:H7 strains (Dean-Nystrom et al. 2000). Neurological signs have been reported in association with a CNS vasculopathy resembling oedema disease (Francis et al. 1989). Similar signs occurred with an *eaeA*-deletion mutant (Tzipori et al. 1987) but not with a Shiga toxin-negative *E. coli* O157:H7 strain (Dean-Nystrom et al. 2000). Surprisingly, the neurological signs were found to be more severe in colostrum-fed suckling piglets than in colostrum-deprived individuals (Dean-Nystrom et al. 2000). Vaccination of pregnant dams against intimin shows a protective effect against intestinal colonisation and the formation of AE lesions in the dam's suckling piglets (Dean-Nystrom et al. 2002).

In summary, piglets inoculated with *E. coli* O157:H7 demonstrate Shiga toxin-independent, *eaeA*-dependent nonbloody diarrhoea associated with AE lesions in the distal ileum and the large intestine. Colostral anti-intimin$_{O157}$ antibody ameliorates the AE lesions and reduces colonisation. Systemic neurological signs and lesions have been induced by Shiga toxin-producing strains. No HC-like clinical signs have been induced.

Rabbit

In contrast to other species, infant rabbits inoculated orally with *E. coli* O157:H7 demonstrate extensive colonisation of the small intestine in addition to the large intestine. AE lesions and nonbloody diarrhoea have been observed (Potter et al. 1985; Pai et al. 1986; Sherman et al. 1988a). Weaned rabbits appear much less susceptible to colonisation by *E. coli* O157:H7 than do infant rabbits (Pai et al. 1986).

Chicken

Persistent *E. coli* O157:H7 infection of the large intestine-like parts of the chicken caeca can be induced by inoculation of one-day old chicks with a high dose (10^9 cfu) of *E. coli* O157:H7. Caecal oedema and attachment of bacteria to the mucosa and were observed, and persistence for up to 88 days p.i. was demonstrated. Clinical signs were not detected (Beery et al. 1985).

1.4.3 Non-O157 STEC

1.4.3.1 Occurrence, detection and significance

Non-O157:H7 STEC are frequently found in association with HC and HUS in the absence of *E. coli* O157:H7, and the detected incidence (10 % in 1987; Bopp et al. 1987) is rising. Indeed, in Australia non-O157:H7 associated HUS cases outnumber those associated with *E. coli* O157:H7 (Robins-Browne et al. 1998), and this has recently become the case in Italy also (Tozzi et al. 2001). Serovars involved include O26:H11, O111:H-, O103:H2, O113:H21, O157:H- and O-rough or untypable (Bopp et al. 1987; Griffin and Tauxe 1991; Willshaw et al. 1992). Strains are usually *eaeA*- and CVD419- (enterohaemolysin) positive (Nataro and Kaper 1998) but the range of detected plasmid sizes are diverse compared with that of pO157 (Willshaw et al. 1992). The experimental inoculation of rabbits with several non-O157:H7 EHEC of diverse serotypes produced diarrhoea with AE lesions (Sherman et al. 1988a). Other evidence for the virulence of non-O157:H7 STEC includes the existence of clusters of such STEC, detected serologically and microbiologically in the human population, plus the detection of Shiga toxin from HUS stools with an accompanying lack of evidence of *E. coli* O157:H7 as sought by serology, culture or other methods (Tarr and Neill

1996). Non-O157:H7 STEC are associated more with sporadic disease than with outbreaks, suggesting a lower virulence or transmissibility than for EHEC O157:H7. The pathogenic and epidemiological features of non-O157 STEC have been reviewed (Griffin and Tauxe 1991; Johnson et al. 1996; Tarr and Neill 1996).

There is evidence of a veterinary species reservoir of non-O157:H7 STEC (Bettelheim 2000). Comparison between human non-O157:H7 EHEC and veterinary STEC shows much overlap of serogroups between bovine, ovine and human isolates (Gonzalez and Blanco 1989; Wray et al. 1993). The O5, O26 and O111 serogroups predominate in STEC isolates from calves (Holland et al. 1999). Serotypes O26:H11 and O111:H-, both prominent among human EHEC, are reported as bovine EHEC (Johnson et al. 1996; Pearson et al. 1999).

1.4.3.2 Pathogenic mechanisms

Virulence factors in non-O157:H7 STEC strains are more variable than with *E. coli* O157:H7, and only Shiga toxin production is found consistently (Johnson et al. 1996). Strains may lack the *eaeA* gene and these are correspondingly FAS- and AE-negative. Such strains have been found in the serovars O91:H21 (McKee et al. 1995a), O-rough (Willshaw et al. 1992) and O113:H21 (Dytoc et al. 1994). Amongst AE EHEC, tyrosine phosphorylation of Tir does occur with some serotypes, including O26:H11 (Kaper 1998), in contrast to *E. coli* O157:H7. An HC-like disease has been reported in eight-month (Pearson et al. 1999) and 19-month (Wada et al. 1994) old heifers, associated with O26:H11 and O15 serovars respectively. Both cases demonstrated AE lesions in the large intestine.

E. coli O111:H- has been reported to cause septicaemic shock in one human patient, and this strain caused similar pathology in gnotobiotic piglets, suggesting that a more invasive phenotype may be present in some EHEC (Tzipori et al. 1988).

Non-adherence and LA-like initial adherence *in vitro* is reported (Willshaw et al. 1992). *E. coli* O113:H21 demonstrates adhesion *in vivo* by an unknown mechanism (Nataro and Kaper 1998). The presence of pO157, as detected by the CVD419 probe, was verified in 10 out of 14 non-O157:H7 EHEC (Ashkenazi et al. 1992) and showed no correlation with adherence to a rabbit ileal epithelial cell preparation. Another study showed a prevalence of pO157 of 77 % in O26:H11 EHEC (Nataro and Kaper 1998). Acid tolerance among non-O157:H7 EHEC is variable and not associated with serotype (Benjamin and Datta 1995). An adherence-associated gene (*efa1*) is present in many EHEC (and EPEC) strains (Nicholls et al. 2000), but it appears to be defective in *E. coli* O157:H7 (Janka et al. 2002).

Thus, there appear to be diverse mechanisms whereby non-O157:H7 EHEC are able to survive in the food chain and colonise the human intestinal tract to deliver Shiga toxin to local tissues and the blood stream.

1.5 Rationale and aims of the present work

The wide distribution and persistence of *E. coli* O157:H7 amongst groups of cattle and sheep is well-documented (present chapter, Section 1.4.2.6), as is the epidemiological connection between *E. coli* O157:H7 in food animals and human disease caused by the organism (present chapter, section 1.4.2.4). One important chain of contamination passes through abattoirs, where animals excreting *E. coli* O157:H7 lead to contamination of carcasses and meat (Elder et al. 2000; Gansheroff and O'Brien 2000; Barkocy-Gallagher et al. 2001). Whilst hygienic practices at abattoirs are effective at limiting contamination of meat (Elder et al. 2000), the low infective dose of *E. coli* O157:H7 for humans and the possible alternative routes of animal to human infection (present chapter, Section 1.4.2.4) make on-farm control of the prevalence of the organism a desirable goal. Several authors have suggested that control strategies based upon reducing excretion rates on farms and at abattoirs are desirable (Gansheroff and O'Brien 2000; Synge 2000; Hancock et al. 2001), and theoretical modelling has indicated that lowering the prevalence of excretion is likely to prove fruitful in the further reduction of abattoir-based contamination of meat (Jordan et al. 1999).

Epidemiological studies indicate that *E. coli* O157:H7 exists apparently as a commensal organism in ruminants, being excreted by a minority of individuals at any one time, in a background of other flora including other *E. coli* strains. In addition, *E. coli* O157:H7 is found in the farm and lairage environment (Midgley and Desmarchelier 2001), including water troughs (Faith et al. 1996; Hancock et al. 1998b; Shere et al. 1998; Sargeant et al. 2000). Reliable detection of the organism in animal faeces is difficult (present chapter, Section 1.4.2.6) and the ecology of the organism on farms and amongst groups of animals is poorly understood. Persistence may be primarily based in the environment, as suggested by some authors (Hancock et al. 1998a), or the organism may reside principally within the intestinal tract of reservoir animals.

Despite experimental work on *E. coli* O157:H7 persistence in the environment (Kudva et al. 1998; LeJeune et al. 2001; McGee et al. 2001) and in animals (present chapter, Section 1.4.2.7), the possible mechanisms of persistence of the organism in animals are still poorly understood. Therefore the present studies aimed to investigate which features of *E. coli* O157:H7 may be important in persistence, primarily concentrating upon LEE-mediated adherence to the intestinal mucosa. Interventions which target specific mechanisms possibly associated with persistence, such as AE adherence, have the potential to reduce the persistent excretion of *E. coli* O157:H7 with the additional benefit of minimising incidental effects upon the rest of the gastrointestinal flora.

Chapter 2 – MATERIALS AND METHODS

2.1 General considerations

2.1.1 Microbiological containment

STEC are classified as hazard category 3 pathogens in the UK. Therefore, any manipulations involving viable STEC were performed within appropriate containment facilities. For *in vitro* characterisation studies, a designated category 3 containment facility at VLA Weybridge was used, and applicable Working Procedures were followed. For *in vivo* studies, animal accommodation and procedures were as described in Section 2.6.

2.1.2 Ethical review and Home Office licensing

All procedures complied with the UK Animals (Scientific Procedures) Act 1986 and were performed under Home Office Licence 70/4987. The Home Office licence was submitted to an independent panel of animal model experts on the VLA ethics review board and scrutinised prior to submission to the Home Office. Additionally, work performed at The University of Bristol, Langford was subjected to ethical review by the University.

2.2 Bacterial strains, growth and enumeration

2.2.1 Strains used

2.2.1.1 *E. coli* O157:H7 strains

The Laboratory of Enteric Pathogens (LEP) of the Central Public Health Laboratory (CPHL) provided toxin- and phage-typed *E. coli* O157:H7 obtained from human sources, with brief clinical details. The VLA provided phage-typed strains from bovine field surveys of *E. coli* O157:H7 excretion (EC218/94, hereafter referred to as 'EC218'; EC222/94, hereafter referred to as 'EC222'), or from an association between human disease and bovine excretion (EC157). The features of each strain examined are summarised in Table 3-A. Strain NCTC 12900, original designation E81186 (Willshaw et al. 1994), was isolated from a diarrhoeic child (Cheasty, T., personal communication) and lacked Shiga toxins. It was genotypically and phenotypically characterised at VLA Weybridge (Dibb-Fuller et al. 2001), when it was shown to encode the following genes: *eaeA* (intimin); *sepABCD* (type III secretion system components); *espA* (LEE secreted protein); *etpD*, *katP*, *hlyA* (virulence plasmid genes, Section 1.4.2.2E). It did not encode either of the Shiga toxin genes *stx1* or

stx2. Studies on HEp-2 and bovine epithelial-like cell monolayers demonstrated that strain NCTC 12900 formed dense microcolonies and intense FAS-positive lesions on all cell types, when compared with the EPEC strain B171 (O111:NM). A quantitative association assay demonstrated similar or greater association of strain NCTC 12900 with all cell types when compared with EPEC strain B171 and with *Salmonella Typhimurium*, but with little invasion ($\leq$ 0.1 %).

2.2.1.2 Reference strains

Four non-O157 *E. coli* strains were used as reference strains in the studies described herein:

- EPEC O111:NM, strain B171. This was originally isolated from an outbreak of diarrhoea in an infant and toddler centre (Paulozzi et al. 1986; Riley et al. 1987).
- STEC O26:K60:H11, strain EC1537. This is an AE strain isolated from an eight-month old heifer with haemorrhagic diarrhoea, in a group of diarrhoeic animals in which O26 AE lesions were identified in the large intestine (Pearson et al. 1999). It was flagellar antigen typed (Gunning et al. 2001) subsequent to the original report.
- AEEC O80:NM, strain 85440. This was isolated from a three-week old calf with diarrhoea associated with AE lesions in the small and large intestines (Pearson et al. 1989). The lesional bacteria were positively immunostained with an antiserum raised against this strain. Subsequently the strain was serotyped (Wales et al. 2000).
- *E. coli* K12, strain DH5α is a plasmid-transformation competent, avirulent, laboratory strain supplied by Invitrogen.

2.2.2 Storage of bacterial strains

Bacterial strains were stored at room temperature on Dorset's egg medium slopes (Appendix 1) in screw-capped bijoux. Subcultures were created by inoculation onto 5 % sheep's blood agar (Appendix 1), and colonies on this medium were examined for uniformity of appearance. New sheep's blood agar subcultures were made from the previous sheep's blood agar plate or Dorset's egg slopes at monthly intervals.

2.2.3 Routine broth culture

Bacterial strains were grown routinely in Luria-Bertani minus glucose (LB-G, Appendix 1) broth. Sterile LB-G broth (1, 3 or 20 ml) was inoculated with a small sweep of colonies from a sheep's blood agar plate of the strain required. Aerobic stationary-phase

broth cultures were generated overnight at 37 °C in a shaking incubator (Innova 4000, 19 mm circular orbit, 175 rpm).

2.2.4 Cultures for bacterial protein analysis in vitro

Strains were grown in 1 ml LB-G broth overnight. Two protein secretion regimes were used:

A. A 200 µl aliquot of broth culture, containing approximately 2 x 10^8 cfu, was inoculated into a tapered 50 ml polythene tube (Falcon) containing 20 ml Dulbecco's modified Eagle's medium (DMEM, with sodium bicarbonate and 4500 mg/l glucose, Sigma) which had previously been equilibrated at 37 °C in 5 % CO_2. The DMEM was then incubated at 37 °C in 5 % CO_2 for five hours.
B. A 10 ml aliquot of broth culture was centrifuged for 10 min at 3490 *g* and the bacterial pellet was resuspended in 20 ml phosphate-buffered saline (PBS, pH 7.2, Appendix 1) at room temperature. This procedure aimed to achieve a final concentration of 5 x 10^8 cfu/ml, similar to that in DMEM after 5 hours incubation. The PBS suspension was then left at room temperature for one hour.

2.2.5 Cultures for studies of the effect of pre-incubation conditions upon cell association

Two preparations were made for each strain, one in DMEM and one in PBS. For the DMEM preparation, 10 µl of an overnight LB-G broth culture was inoculated into 20 ml of DMEM, which had been equilibrated at 37 °C and 5 % CO_2. The inoculated DMEM was incubated for three hours in a 37 °C, 5 % CO_2 incubator and then 1 ml of it was added to a washed HEp-2 cell monolayer. Alternatively, for the PBS preparation, 150 µl of overnight LB-G broth culture was added to 850 µl of PBS and the bacterial cells were washed by centrifugation and resuspension in 1 ml PBS. The bacterial suspension was left for one hour at room temperature and was then centrifuged, resuspended in 10 µl of PBS and added to 990 µl of DMEM on a freshly-washed HEp-2 monolayer.

2.2.6 Cultures for the intestinal loop experiments

(Dr. A. Cookson prepared all cultures)

The strains were grown routinely overnight and 1 ml of broth culture was harvested by centrifugation at 13 400 *g* on a bench-top centrifuge (Microcentaur, MSE). The bacterial pellet was resuspended in 4 ml PBS to give a viable count of approximately 2.5 x 10^8 to 5 x 10^8 cfu/ml. Alternatively, 200 µl of a routine overnight broth culture was inoculated into

20 ml pre-warmed DMEM and incubated for six hours at 37 °C in 5 % CO_2 to give a viable count approximately equivalent to that in the PBS-suspended preparation. The total viable count (TVC) of bacteria in inocula was determined for the final two experiments (lambs 3 and 4).

2.2.7 Cultures for oral inoculation experiments

(Mrs. C. Hayes or Dr. A. Cookson prepared all cultures)

The four-strain mixed inoculum was made by mixing a 2.5 ml aliquot of an overnight LB-G broth culture of each of the four strains together and making the mixture up to 100 ml with PBS.

Single-strain inocula were prepared by one of three methods:

a) A 10 ml aliquot of an overnight culture was made up to 100 ml with PBS.
b) An 8-hour LB-G broth culture was given orally without further processing.
c) A 60 ml overnight LB-G broth culture was sedimented by centrifugation and resuspended in 50 ml PBS.

Aliquots of each inoculum preparation were taken for TVC determination. Each animal was inoculated orally with 10 ml of the appropriate inoculum.

2.2.8 Determination of total viable count (TVC)

A technique based on that described by Miles and Misra (1938) was used for the determination of TVC. A 20 µl aliquot was removed from the bacterial suspension to be measured and was diluted tenfold by addition to 180 µl of PBS in a polystyrene microtitre plate well. A serial tenfold dilution series was prepared in PBS on the microtitre plate. Depending upon the likely viable count, dilutions of 10^4- to 10^6-fold were prepared. From each of the three or four most dilute suspensions, three 20 µl aliquots were plated onto labelled, partially-dried nutrient agar plates for overnight culture. After incubation, the least dilute suspension for which clear, separated colonies could be counted was used for colony counting, and an average number of colonies per 20 µl spot was determined from the three spots. The viable count of the original suspension was derived by multiplication using the dilution factor.

2.2.9 Recovery and enumeration of bacteria from experimental animals

Most samples were analysed immediately after being taken, as follows. Faeces and intestinal contents (1 g) and tissue samples (2.5 g) were collected, placed in 9 and 22.5 ml

volumes of BPW (pH 7.2, Oxoid) respectively and homogenised in a stomacher. Rectal swabs were placed in 10 ml BPW and vortex-mixed. Samples were weighed in plastic petri dishes on a portable balance. Some faecal and intestinal contents samples weighed less than 1 g due to constraints of the sampling procedure.

A Most Probable Number approach was used to determine, semi-quantitatively, the *E. coli* O157:H7 count in each sample cultured. Each homogenate was diluted 10- or 100-fold serially in BPW and then each dilution was incubated for six hours at 37 °C before 1 ml of the supernatant was processed via IMS, according to the manufacturer's instructions. For IMS, 20 µl of magnetic beads coated in polyclonal antisera raised against the O157 lipopolysaccharide (Dynabeads anti-*E. coli* O157, Dynal) were mixed with the 1 ml sample in a 1.5 ml microcentrifuge tube (Eppendorf) on an inclined rotator at 30 rpm for 30 min at room temperature. Each tube was placed in a magnetic separator rack (Dynal) for 5 min and the rack was then gently inverted three times, to concentrate the magnetic beads against the side of the tube adjacent to the external magnet. The liquid was removed from the tube with a pipette and replaced with 1 ml of PBS containing 0.5 % Tween 20 (Sigma). The tube was removed from the magnet, gently inverted four or five times to mix the beads with the PBS-Tween, and replaced in the magnetic rack. The beads were washed by this procedure twice more and then resuspended in 100 µl PBS.

In experiments involving the four-strain mixed inoculum, 25 µl aliquots of the final suspension of beads were spread onto separate nalidixic acid (nal; 15 µg/ml), rifampicin (rif; 150 µg/ml), streptomycin (str; 25 µg/ml) or nal (15 µg/ml) plus rif (150 µg/ml) - supplemented CHROMagar O157 (CHROMagar) plates. To differentiate between single and double antibiotic resistance marked *E. coli* O157:H7 strains, 10 colonies from nal- and rif-supplemented plates were subcultured onto rif and nal plates respectively. In the experiments using a single inoculum strain, all of the final suspension was spread onto nal- or rif-supplemented or antibiotic-free CHROMagar O157 plates, according to the resistance marking of the strain involved. The final suspension volume was 100 µl for the antibiotic resistant strains and 200 µl for the unmarked strain. The aim of the increased volume for the latter was to spread out the background flora carried over from the IMS procedure, which would not be suppressed by the antibiotic-free culture medium.

Where sample processing was to be delayed, 2.5 g samples were collected into 22.5 ml PBS containing 15 % glycerol, frozen and stored on dry ice overnight. The frozen preparations were then thawed at room temperature and a 2.5 ml aliquot was mixed with 22.5 ml BPW. This mixture was then serially diluted, incubated and subjected to the IMS and culture procedures described above.

For pre-inoculation screening of lambs for the excretion of *E. coli* O157, the IMS procedure described above was used, without the preparation of a dilution series before pre-

enrichment. The IMS beads were spread onto a CHROMagar O157 plate without antibiotic supplements.

2.2.10 Analysis of bacteria cultured from experimental animals

The serogroup of lilac-coloured colonies recovered on CHROMagar O157 was checked by O157-specific latex agglutination (LAgg; *E. coli* O157:H7 Test, Oxoid), following the instructions of the manufacturer. Any lilac colonies which produced a negative result with LAgg were subcultured and retested, and persistently negative lilac cultures were stored on Dorset's egg medium for further analysis if required.

Serogroup determination and Verocytotoxicity testing of selected strains was performed at the VLA *E. coli* typing unit using standardised multiwell plate agglutination tests and a polymyxin B-Verocell assay respectively. PCR detection of *eaeA*, *stx1* and *stx2* was also performed on selected strains, either at the VLA *E. coli* typing unit or as described in Section 2.3.

2.3 Genotypic characterisation of experimental strains

2.3.1 Preparation of genomic DNA

Template DNA for PCR reactions was prepared by phenol-chloroform extraction (Ausubel et al. 1987). Bacteria, grown overnight in 3 ml LB-G broth, were sedimented into the bottom of a single 1.5 ml microcentrifuge tube (Eppendorf) by three cycles of centrifugation of 1 ml for 3 min at 13 400 *g* in a bench-top centrifuge: the supernatant was discarded and further broth suspension was added after each of the first two spins. The bacterial pellet was resuspended in 400 µl of Tris-EDTA buffer, pH 8.0 (Appendix 1), to which 50 µl of Proteinase K (20 mg/ml, Sigma) was added. The mixture was incubated at 55 °C for 30 min and then the bacterial cells were lysed by the addition of 50 µl 10 % w/v sodium dodecyl sulphate (SDS 99 %, Sigma). Incubation continued at 55 °C until the suspension cleared (approximately 2 min). The lysed cell suspension was then added to a 1.5 ml Phase Lock Gel (PLG) Light tube (5'→ 3') which previously had been centrifuged briefly to deposit the PLG at the bottom of the tube. An approximately equivalent (500 µl) volume of phenol chloroform mixture (25 phenol : 24 chloroform : 1 isoamyl alcohol, buffered to pH 8.0 with Tris, Sigma) was added to the PLG tube. The fluids were emulsified, by gentle inversion until the mixture turned milky white, and were then separated by centrifugation as above for 15 min. The aqueous (topmost) layer overlying the PLG/phenol chloroform interface was removed to a fresh PLG tube and 600 µl of phenol chloroform was

added to this tube. The fluids were mixed and separated by centrifugation as described above.

The aqueous layer (approximately 500 µl) was then decanted into a new 1.5 ml microcentrifuge tube to which isopropyl alcohol (1 ml, Sigma) was added. The tube contents were gently mixed by inversion for 1 min, until a fluffy white DNA precipitate was formed. The precipitate was sedimented by centrifugation as above for 5 min and the supernatant was discarded. The DNA pellet was washed with 1 ml of 70 % ethanol and the tube was centrifuged as above for 3 min. The supernatant was discarded and the DNA pellet was allowed to partially dry by evaporation of the ethanol at room temperature for 15 min, before being gently resuspended in 250 µl double-distilled sterile water and allowed to dissolve overnight at 4 °C.

2.3.2 Polymerase Chain Reaction (PCR)

PCR primer sequences were obtained from quoted references (Table 2-A), or were designed by Dr A. L. Cookson at VLA Weybridge using a DNASTAR computer program, from published GenBank sequences (NCBI) using the accession numbers quoted in Table 2-A. The *eaeA* primers amplified a fragment from the 5' (non-variable) end of the intimin gene. The *stx1* primers amplified a fragment spanning the A- and B-subunit genes and the *stx2* primers amplified a fragment of the A-subunit gene. The enterohaemolysin primers amplified a fragment spanning the EHEC-*hlyC* and -*hlyA* genes. Primer sequences and the expected product sizes are given in Table 2-A.

To minimise the possibility of foreign DNA contamination, work surfaces were sprayed and wiped with 70 % ethanol and plugged (non-aerosol) pipette tips (Anachem) were used. Control negative PCR reactions lacking template DNA and positive reactions containing DNA with the target sequence were included in all batches of reactions.

PCR reactions (Saiki et al. 1985) were carried out in a total volume of 50 µl, in thin-walled PCR tubes (Sigma). The Cambio PCR Optimization Kit MasterAmp 2x PCR buffers A, B, and C were used. These buffers all contain: 100 mM Tris-HCl (pH 8.3), 100 mM KCl and 400 µM of each deoxyribonucleoside triphosphate (Adenine, Thymidine, Cytosine and Guanidine). Buffers A, B and C contained 3.0, 5.0 and 7.0 mM $MgCl_2$ respectively. Three reaction mixtures, containing buffer A or B or C, were prepared for each strain tested in order empirically to provide the optimal magnesium concentration for each amplification. Where previous experience had demonstrated an optimal buffer, or no difference between buffers for a particular product, only one buffer was used subsequently. Each reaction mixture was prepared on ice by addition of reagents in the following order:

i. 17.5 or 21.5 µl double distilled sterile water, depending upon whether the PCR reaction was single or multiplex (18.5 or 22.5 µl in negative control tube)
ii. 25 µl MasterAmp™ 2x PCR buffer A, B, or C
iii. 1 µl (40-60 pmol) each of forward and reverse primers for each product, i.e. two primers for single PCR, six primers for multiplex PCR
iv. 0.5 µl *Thermus aquaticus* (*Taq*) DNA polymerase (Promega)

The tube was then vortex-mixed, the entire reaction mixture was relocated to the bottom of the tubes by brief centrifugation, and two drops of sterile mineral oil (Sigma) were added.

v. Template DNA (1 µl of preparation) was introduced into the aqueous phase by pipetting through the oil.

The sealed tube was then vortexed and centrifuged briefly, to mix the contents.

Amplification was then performed in a Trio thermal cycler (Biometra) using a 'touchdown' program with high early annealing temperatures to optimise specificity. The program used was as follows:

i. Initial denaturing at 94 °C for 5 min (tubes added to pre-heated block)
ii. High-stringency annealing at 68 °C for 1 min
iii. Extension at 70 °C for 1 min per thousand base-pairs in the product
iv. Denaturing at 94 °C for 1 min

Steps (ii) through (iv) were then repeated six times, reducing the annealing temperature by 2 °C each time, to a final 56 °C. This was followed by:

v. Annealing at 56 °C for 1 min
vi. Extension at 70 °C for 1 min per thousand product base pairs
vii. Denaturing at 94 °C for 1 min

Steps (v) through (vii) were then repeated 28 times, followed by:

viii. Final holding at 4 °C until products were removed for electrophoresis

PCR products were separated by electrophoresis within 12 hours. Agarose gels (0.8 %, Appendix 1) were cast in horizontal tanks (BioRad) and immersed in TAE buffer. Reaction products (10 µl) were mixed with 4 µl of loading buffer (Appendix 1) and the 14 µl mixtures were loaded into gel wells. Molecular mass markers (1 Kb Plus Ladder, Life Technologies) were loaded into adjacent lanes. Electrophoresis was performed at 160 V until the bromophenol blue dye front approached the end of the gel.

Separated products were rendered fluorescent by soaking the gel in an ethidium bromide solution (8 µg/ml in TAE buffer) for 5-10 min. The gel was then rinsed in distilled water and

examined for fluorescence on an ultraviolet transilluminator (Syngene). The appearance of each gel was recorded photographically via video camera and monochrome printer.

Table 2-A: PCR primers used for strain characterisation

Target gene	Primer sequences	Expected product size (bp)	Accession no. / ref. (Source serotype)
eaeA	F: TGC GGC ACA ACA GGC GG R: CGG TCG CCG CAC CAG GAT TC	628	Z11541 (O157:H7)
eaeA, ⇨ subtype	F: TGC GGC ACA ACA GGC GG R: GCT CAC TCG TAG ATG ACG GCA AGC G	2135	AF116899 Oswald et al. 2000 (O103:H2)
stx1	F: GGC AGA TGG AAG AGT CCG TGG GAT TAC GC R: CAC AAT CAG GCG TCG CCA GCG CAC TTG CT	178	Z36900 (O111:H-)
stx2	F: CCA CAT CGG TGT CTG TTA TTA ACC ACA CC R: GCA GAA CTG CTC TGG ATG CAT CTC TGG TC	374	X61283 (O157:H7)
espP	F: TTG CGA AAA ATG GCG GAA CTC R: GCT GAC GGG GCA TTG ACT G	1146	X97542 Brunder et al. 1996 (O157:H7)
katP	F: CTT CCT GTT CTG ATT CTT CTG G R: AAC TTA TTT CTC GCA TCA TCC	2125	Brunder et al. 1996 (O157:H7)
hlyA-C	F: GCT ATG GGC CTG TTC TCC TCT G R: TGT CTT GCG TCA TAT CCA TTC TCA	1779	X86087 (O157:H7)
etpD	F: CGT CAG GAG GAT GTT CAG R: CGA CTG CAC CTG TTC CTG ATT A	1061	Y09824 (O157:H7)
rpoS	F: ATG AGT CAG AAT ACG CTG AAA GTT CAT GAT T R: AGA CTG GCC TTT CTG ACA GAT GCT TAC TTA	1019 (entire gene)	Waterman et al. 1996 (DH1, non-O157)
bfpA	F: C GGC GGA TCC* TGG TTT CTA AAA TCA TGA ATA AG R: C GGC AAG CTT† CTT CAT AAA ATA TGT AAC TTT AT	578	Z12295 (O127:H6)

Underlined sequences are non-hybridising extensions incorporating **Bam*H I and †*Hin*d III restriction sites.

2.4 Generation of spontaneous antibiotic resistant mutants

Antibiotic resistant bacterial mutants were selected, to aid recovery from inoculated animals and to permit subsequent identification of strains. Nalidixic acid, streptomycin, rifampicin and spectinomycin (spec) were used. Strains were spread-plated (200 µl from overnight cultures in LB-G broth) on nutrient agar plates containing nal (15 µg/ml), str (25 µg/ml), rif (150 µg/ml) or spec (100 µg/ml). After overnight aerobic culture at 37 °C, spontaneous antibiotic resistant colonies present on these plates were subcultured onto the

same media three further times, then maintained on Dorset's egg medium slopes at room temperature, and subcultured onto sheep's blood agar plates prior to any procedures.

A double-marked nal^{r}rif^r strain was generated by sequential culture on nal (15 µg/ml) and rif (150 µg/ml) -supplemented nutrient agar plates.

2.5 Phenotypic characterisation of experimental strains

2.5.1 Species confirmation and serotyping

The species of presumptive *E. coli* was confirmed at the VLA *E. coli* typing unit by growing the isolate in O-nitrophenol-D-galactopyranoside (ONPG) broth to test for β-galactosidase activity, indicating a lactose fermenter, and by growing it on Tryptone Bile Agar (TBA) for an indole spot test. If the isolate was a non lactose-fermenter or indole test negative or failed to grow on TBA, an API 20E (bioMérieux) strip test for Enterobacteraceae identification was performed.

Somatic antigen ('O') serotyping was done by standard microtitre agglutination tests on boiled and autoclaved broth cultures, initially against a panel of 48 antisera raised in rabbits. Further antisera were used if necessary. Capsule detection and typing was performed with a slide agglutination method using homologous 'O' antisera and test capsular antisera.

2.5.2 Survival at acid pH

2.5.2.1 Inorganic acid

For each strain, the TVC of an overnight LB-G broth culture was determined. A 10 µl aliquot of the broth culture, containing approximately 10^7 cfu, was diluted in 990 µl of PBS, and 30 µl of this was added to 2970 µl of LB-G broth which had been acidified to pH 2.5 by the addition of concentrated hydrochloric acid. Thus, an initial concentration of approximately 10^5 cfu/ml was present in the acidified broth. The broth was incubated aerobically at 37 °C with shaking, (Innova 4000 incubator, 19 mm circular orbit, 175 rpm), for 2.5 hours.

To enumerate the TVC after incubation, the bacterial mass was sedimented into the bottom of a single 1.5 ml microcentrifuge tube (Eppendorf) by two cycles of centrifugation for 3 min at 13 400 *g* in a bench-top centrifuge, with the supernatant discarded after each spin. The bacterial pellet was resuspended in 200 µl of PBS and a TVC determined. Using the TVC of the initial inoculum (derived from the overnight broth culture) and of the post-incubation acidified broth (derived from the resuspended bacterial pellet), a percentage survival was calculated.

2.5.2.2 Volatile fatty acids (VFA)

Acetic, propionic, butyric and valeric acids (Sigma) were mixed in a molar ratio of 77:10:10:3, to a total VFA concentration in LB-G broth of either 100 mmol/l or 150 mmol/l. Three aliquots of each mixture, plus three aliquots of control LB-G broth without added VFA, were adjusted to pH 6.0, 6.5 and 7.0 respectively, using concentrated aqueous sodium hydroxide and hydrochloric acid. Thus, three added VFA concentrations (0, 100 and 150 mmol/l) were prepared at each of three acidity levels. Each VFA plus pH combination was filter-sterilised using a 0.2 µm syringe filter (Minisart filter, Sartorius) and equilibrated overnight at 37 °C in an anaerobic gas jar (Becton-Dickinson 'BBL GasPak' anaerobic system with palladium catalyst).

Each test strain was inoculated singly into each VFA plus pH combination. An initial 100-fold dilution of an overnight LB-G broth culture of each strain was prepared in PBS, and 10 µl of this was inoculated into 990 µl of the VFA mixture, in a 1.5 ml screw-capped microcentrifuge tube with a hole in the cap to permit gas exchange. A 20 µl aliquot of the inoculated VFA mix was withdrawn immediately and the TVC determined. All inoculated microcentrifuge tubes were then placed in anaerobic gas jars and incubated at 37 °C. The gas jars were opened at four or five hours p.i. and 20 µl aliquots were taken from all microcentrifuge tubes for TVC determinations, then the jars were resealed with fresh anaerobic packs and final samples for TVC were taken at eight hours p.i.

2.5.3 Cross-streaking and growth in broth culture

(Dr. A. Cookson performed all work)

The growth of each strain in LB-G broth at 37 °C with shaking, (Innova 4000 incubator, 19 mm circular orbit, 175 rpm) was examined by monitoring optical density (OD). For each antibiotic resistance marked strain, 20 ml of a 10^6-fold dilution of an overnight stationary phase broth culture, approximating 10^3 cfu/ml, was prepared using fresh, warmed LB-G broth. This culture was incubated aerobically in 50 ml plastic tubes (Falcon) at 37 °C with shaking. At hourly intervals for nine hours, a 1 ml aliquot was removed and its OD at 600 nm was read in a spectrophotometer (Biotec UV1101, WPA).

Interactions between strains were assessed by mixed batch culture and cross-streaking. A low-density mixed-strain LB-G broth culture (20 ml) was made up from overnight broths of each antibiotic resistance marked strain, to a concentration of about 10^3 cfu/ml for each strain, as for the single-strain growth curves. The mixture was incubated as described above for the single strains. At hourly intervals for nine hours, an aliquot of the culture was

removed and TVC were determined for each strain using antibiotic-supplemented culture plates.

For cross-streaking, a nutrient agar plate was inoculated with intersecting lines of each antibiotic resistance marked strain, such that each strain intersected every other strain. The plate was incubated aerobically at 37 °C overnight.

2.5.4 Analysis of bacterial proteins

2.5.4.1 Preparation of concentrated secreted protein solutions

Bacterial strains grown overnight in LB-G broth were inoculated into DMEM and PBS and incubated, as described in Section 2.2.4. A 1 ml aliquot was removed from each of the cultures for processing in the intimin expression assay (Section 2.5.4.2). A 20 µl aliquot was removed from this for a TVC determination. The remaining approximately 19 ml of DMEM or PBS preparation was centrifuged at 3490 *g* for 10 min in a refrigerated centrifuge (Megafuge 1.0 R, Heraeus) at 4 °C. The supernatant was removed, using a 20 ml syringe, and filter-sterilised through a 0.2 µm filter (Minisart filter, Sartorius) into a sterile tapered polythene tube (Falcon). To demonstrate sterility before removal from the category 3 containment laboratory, a 100 µl aliquot of each the filter-sterilised preparations was spread onto a sheep's blood agar plate, incubated at 37 °C overnight, and examined for bacterial growth. The remaining liquid was stored at 4 °C and, following the demonstration of sterility as above, were removed to a hazard category 2 containment laboratory.

Secreted proteins were concentrated by centrifugal ultrafiltration. Most (17 ml) of each sterile DMEM or PBS preparation was pipetted into the upper chamber of a Vivaspin 20 concentrator (Vivascience) containing an ultrafiltration membrane with a molecular weight cut-off (MWCO) of 5000 daltons. The Vivaspin apparatus (upper chamber separated from a lower chamber by the membrane) was then centrifuged at 3500*g* in a refrigerated centrifuge for 10-20 min, until the sample in the upper chamber had been reduced in volume to approximately 500 µl. This concentrated macromolecule solution was then decanted by pipette and either analysed immediately or stored frozen at -20 °C. The secreted proteins were separated and detected by SDS-PAGE, silver staining and Western blotting as described in Section 2.5.4.3.

2.5.4.2 Intimin expression preparations

Two protocols were used:

A. An overnight LB-G broth culture (200 µl) was inoculated into 20 ml of warmed DMEM (4500 mg/l glucose) and incubated for five hours at 37 °C in 5 % CO_2. The culture was

sedimented by centrifugation at 3490 *g* for 10 min, the bacteria were resuspended in 200 µl of double-distilled sterile water, and heated in a boiling water bath. The protein concentration of the lysed samples was assayed using a bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce), with 10 µl sample volumes in a 96-well polystyrene microtitre plate (Nunc), according to the manufacturer's instructions. The absorbance of the reaction mixtures (two per strain lysate) was read at 550 nm in a plate reader (Revelation, Dynex). The calculated absorbance of each lysate was the mean of the two readings obtained, minus the mean of the 10 control negative reaction mixtures.

B. Intimin expression studies were performed on the same DMEM and PBS preparations that were used for the secreted protein studies in Section 2.5.4.1. Following incubation a 1 ml aliquot in a microcentrifuge tube, less 20 µl used for a TVC, was centrifuged at 13 400 *g* in a bench-top centrifuge for 3 min and the supernatant was discarded. The bacteria were resuspended in 100 µl of SDS-PAGE loading buffer and heated to 99.9 °C for 5 min in a thermal block. The preparations were stored frozen.

Intimin preparations were separated and analysed by Western blotting as described in Section 2.5.4.3B.

2.5.4.3 Separation and detection of bacterial proteins

A. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and dtection by silver or Coomassie staining

A 12 % polyacrylamide mini-gel with 5 % stacking gel (Appendix 1) was prepared and immersed in SDS-PAGE running buffer in an electrophoresis tank (BioRad) An aliquot (10 to 30 µl) of concentrated macromolecule solution (Section 2.5.4.1) was mixed with SDS-PAGE loading buffer in a ratio of 1:1 (buffer i.) or 4:1 (buffer ii.). An aliquot (10 µl for silver stained gels, 30 µl for Coomassie stained gels) of each preparation was then loaded into a separate well of the stacking gel. A molecular weight marker (Rainbow, Amersham) was included as 10 µl of a 1:50 dilution of the supplied stock mixture. Electrophoresis was performed at 60 V until the bromophenol blue dye marker passed into the separating gel, then at 100 V thereafter, until the dye marker approached the bottom of the gel.

The gel was washed in deionised water and then either fixed and silver stained with the PlusOne silver staining kit (Amersham) according to the manufacturer's instructions, or stained with Coomassie stain. For the latter, the gel was immersed in Coomassie reagent (Appendix 1) overnight, followed by immersion in destaining solution (Appendix 1) for 24 hours, with white tissue paper being used to remove from the destaining solution the stain

extracted from the gel. Protein band patterns were scanned in a flatbed scanner and stored as digital images.

B. Western blotting

SDS-PAGE was performed, loading the gel as described above, but using 20 µl (loading buffer i) or 30 µl (loading buffer ii) of the sample/loading buffer mix and using 10 µl of undiluted Rainbow marker mix. In the case of intimin preparations made using Protocol A (Section 2.5.4.2), the technique was altered to correct for protein content of the samples such that an equal amount of protein was loaded into each well of the gel. This was achieved by adding an amount of each lysate preparation in inverse proportion to its protein concentration (as determined by the BCA assay) to 10 µl of loading buffer. Thus, the sample with the lowest calculated absorbance was added at 10 µl per well, and those with higher absorbances were added in lower proportions, as the BCA assay demonstrates a linear relationship between protein concentration and absorbance over the assayed protein range of 1-2 mg/ml. The sample and loading buffer mix were heated to 99.9 °C in a heating block for 5 min before loading.

Electrophoresis was performed as described for the silver-stained gels. When this was completed, the separated proteins were transferred to a nitrocellulose membrane (Sartorius) by transfer using an electrophoresis tank (BioRad), using a gel-nitrocellulose sandwich suspended in CAPS buffer (Appendix 1). The transfer electrophoresis was run at 300 mA for one hour.

The EspA protein was detected on the nitrocellulose membrane of secreted protein preparations using a rabbit anti-EspA polyclonal antibody, raised against HIS-tagged recombinant EspA from *E. coli* O157:H7 strain EC157 (Table 3-A), produced at VLA Weybridge (Appendix 2). The membrane was initially blocked against non-specific protein binding sites by incubation overnight in 3 % skimmed milk powder (Marvel, Premier Brands) in Tris-buffered saline (TBS, Appendix 1). After washing in TBS containing 0.05 % Tween 20 (Sigma), the membrane was incubated for 45 min at 37 °C in anti-EspA antibody diluted 1:500 in 3 % Marvel in TBS. Following washing in TBS-Tween, the membrane was again incubated for 45 min at 37 °C in goat anti-rabbit peroxidase conjugate (Sigma) diluted 1:2000 in 3 % Marvel in TBS. The bound antibody-peroxidase complex was detected using amino ethylcarbazole (AEC) substrate (Appendix 1), yielding a red reaction product after a few minutes. The development was monitored by eye and stopped by rinsing with deionised water. The dried membranes were scanned on a flatbed scanner and images were stored digitally.

The nitrocellulose membrane of intimin preparations was probed, as described above, with an anti-intimin primary antibody raised in a rabbit against the C-terminal of the γ-intimin molecule from *E. coli* O157:H7 strain A84 (Woodward et al. 1999), expressed in a

recombinant *E. coli* as a HIS-tagged protein (Appendix 2). The resultant stained membranes were dried and scanned on a flatbed scanner and images were stored digitally.

2.5.5 Association of bacterial strains with cultured cells

2.5.5.1 Preparation of cell monolayers

The HEp-2 (human cervical carcinoma derivative, ECACC; Moore et al. 1955; Chen 1988) cell line was supplied by the VLA Weybridge tissue culture unit. Cells were maintained in 75 cm^2 polystyrene flasks with 20 ml complete tissue culture medium (Appendix 1), at 37 °C in 5 % CO_2. They were subcultured every three days by detaching and disrupting the cell monolayer using a 10 min incubation with 3 ml of a proteolytic / EDTA solution (Accutase, ICT), resuspending the cells and discarding 80 % of the suspension. The remaining 20 % of cell suspension was made up to 20 ml with fresh complete medium and re-introduced into a new 75cm^2 flask.

Confluent monolayers of cells in 24-well polystyrene plates (Nunc), either with or without 13 mm diameter glass coverslips, were prepared from confluent stocks in flasks. The flask monolayers were disrupted with 3 ml Accutase as above, and the resulting suspension was diluted with 10 ml complete medium. A viable cell count was performed in a Neubauer-type counting chamber, using 0.1 % erythrosin B (Sigma) to stain non-viable cells. Complete medium was added to adjust the cell suspension to 2 x 10^5 cells/ml and 1 ml of this suspension was added to each well of a 24-well plate. A microscopic check for confluence was made after incubation for 48 hours.

Bovine intestinal cell cultures (ileum, colon and rectum) were obtained from frozen stocks prepared by Dr M. Dibb-Fuller from a neonatal calf (Dibb-Fuller et al. 2001). Frozen vials containing approximately 10^7 cells in Cell Culture Freezing Medium (Gibco) were retrieved from storage in liquid nitrogen and thawed at room temperature. The cells were sedimented by centrifugation at 405 *g* for 10 min and the supernatant was decanted. The cells were resuspended in 1 ml of complete medium and were grown for 72 hours at 37 °C in 5 % CO_2. At this point the cells were subcultured by trypsinisation and sowing, at 2 x 10^5 viable cells per well, into 24-well polystyrene plates. Cells were grown either on the bottom of each well or on a 13 mm diameter glass coverslip within each well, as described for HEp-2 cells. Seeded wells were incubated at 37 °C, 5 % CO_2, for 48 to 72 hours before checking for confluence by light microscopy and then their use in assays.

2.5.5.2 Quantitative association assay

The assay was based upon published methods (Sherman et al. 1987). Bacterial strains were grown overnight in LB-G broth. Cell monolayers grown in wells without coverslips, as described in Section 2.5.5.1, were washed with three changes of 2 ml of Hank's balanced salt solution (HBSS, Sigma), warmed to 37 °C. The monolayers were covered with 990 µl incomplete medium (Appendix 1) pre-equilibrated in 5 % CO_2 at 37 °C. Mannose was omitted from the incomplete medium in the bovine cell culture assays as *E. coli* O157:H7 does not usually express mannose-sensitive fimbriae (Section 1.4.2.2D). For each strain culture a 10 µl aliquot, containing approximately 10^7 cfu, was added to two to four wells. The plates were placed in a humidified 5 % CO_2 incubator at 37 °C for three hours. The incomplete medium was drawn off each well and the monolayers were washed three times with warmed HBSS. After the final wash had been removed, 1 ml of 1 % Triton X-100 detergent (Sigma) was added to each well and the monolayers were detached and disrupted by placing a magnetic stirring flea in each well and agitating the contents on a magnetic stirrer for 10 min. The suspensions resulting from this procedure were used for TVC determination.

2.5.5.3 Giemsa-stained preparations

Bacterial strains were grown overnight in LB-G broth. Confluent monolayers were grown on 13 mm diameter glass coverslips in 24-well plates, as described in Section 2.5.5.1, and were washed, covered with 990 ml of incomplete medium and inoculated with 10 µl of broth culture, as described in Section 2.5.5.2. Monolayers were incubated at 37 °C in a humidified 5 % CO_2 atmosphere for three or six hours. The six-hour monolayers were washed three times with HBSS at three hours p.i. and fresh incomplete medium was added subsequently. After incubation the monolayers were washed three times with HBSS and then fixed for 30 min with 3 % neutral buffered formalin (Sigma). After washing with double-distilled sterile water the cells were stained with 10 % Giemsa stain (Sigma) for one hour, washed three times with double-distilled water, and differentiated in 1 % acetic acid (Sigma) for 1 min. After a final wash, the coverslips were removed from the wells with forceps, air-dried and mounted monolayer-side down on glass microscope slides using DPX mountant (R. A. Lamb). Slides were examined by light microscopy using 40x (dry) and 100x (oil immersion) objectives.

2.5.5.4 Fluorescence actin staining (FAS)

FAS was performed on confluent monolayers grown on 13 mm diameter glass coverslips in 24-well plates, as described in Section 2.5.5.1. An aliquot (10 µl) of each strain, grown in

LB-G broth, was inoculated into a separate well containing 990 µl incomplete medium, and incubated for either three or six hours, with washing in the latter case, as described for Giemsa preparations. After incubation each monolayer was washed four times with 2 ml warmed PBS and then fixed for 20 min with 1 ml of 3 % neutral buffered formalin (Sigma). After washing three times with PBS the cells were permeabilised in 0.1 % v/v Triton X-100 detergent (Sigma) for 4 min. After three further PBS washes, the cells were covered with 0.5 µg/ml fluorescein isothiocyanate-phalloidin (Phalloidin–FITC labelled, Sigma) for 20 min, in the dark. After three final washes in PBS the coverslips were removed from the wells, dried and mounted monolayer-side down on glass microscope slides using DPX mountant. Slides were stored at room temperature in the dark and examined under incident fluorescence illumination with a Zeiss Axiovert 25 fluorescence microscope equipped with a FITC fluorescence filter, using a 100x oil-immersion objective.

Comparison of DMEM- and PBS-prepared strains

The TVC of both DMEM- and PBS-prepared inocula (Section 2.2.5) were enumerated on a 20 µl sample taken from the wells immediately after inoculation of the monolayers. A single six-hour incubation, with washing at three hours, was performed and the cells were then subjected to FAS.

2.5.5.5 Inoculation of monolayers for electron microscopy

Comparison of strains

Monolayers were inoculated, incubated and washed as described in Section 2.5.5.3. The infected monolayers were fixed with 1 % glutaraldehyde overnight.

Comparison of culture conditions

Monolayers were inoculated with bacteria pre-incubated in DMEM or PBS, as described in Section 2.2.5, and incubated and washed as described in Section 2.5.5.3. After washing with HBSS, the monolayers were fixed with 1 % glutaraldehyde overnight.

2.6 *In vivo* studies

2.6.1 Housing, workspaces and protection of personnel

Neonatal lambs inoculated with non Shiga toxin-producing *E. coli* O157:H7 (strain NCTC 12900) were housed indoors in an experimental animal containment facility at the University of Bristol, Langford site. Inoculation, feeding, tissue and faecal sampling, and

euthanasia were carried out in the same facility using category 2 disinfection and personal protection procedures.

Neonatal lambs inoculated with STEC O157:H7 and all six- to 11-week old lambs were housed indoors under category 3 containment in the Aerobiology Unit, University of Bristol, Langford. This comprised an animal pen linked via a hatch to a chamber and a workstation (Figure 2-a, i). The latter two facilities were both located within an isolator, with a half-suit permitting manual procedures within the chamber (Figure 2-a, ii) and arm portals for manipulations on the table workstation (Figure 2-a, iii). Thus, potentially contaminated material or animals could be passed into the chamber and/or workstation and manipulated by operators working from outside the isolator.

Six-month old lambs were housed in a category 3 containment building at VLA Weybridge. For tissue sampling they were transferred to a post mortem room for euthanasia and dissection. Category 3 containment personal protection, including the use of high-efficiency EN 143 Class P3 respirator helmets, plus disinfection procedures using Farm Fluid S (Antec) at a 1:100 dilution and 70 % ethanol, were used.

Surgical and sampling procedures for intestinal loop experiments were performed in a category 3 containment animal house using appropriate protective clothing and disinfection procedures, as for the oral inoculation experiments. Respirator helmets were worn during times when STEC were potentially present as aerosol, i.e. during inoculation and removal of intestinal loops.

2.6.2 Clean sampling technique

Two approaches were used to gain access to tissues:

i. Lambs were euthanased and rapidly dissected, using a primary operator (the Author) and several assistants, in an open post mortem room at VLA Weybridge.
ii. Terminal anaesthesia was used to maintain tissue bloodflow for most intestinal samples until removal from the animal. This technique was employed using a single operator (the Author) with the isolation facility at the University of Bristol Aerobiology Unit.

Samples were taken as follows:

i. The body surface was disinfected using surgical scrub (Hibiscrub, SSL) before opening body cavities. Where there was substantial fleece, i.e. in the weaned lambs, this was sheared off over the right flank before inoculation. A clean surgical drape was used to isolate exteriorised viscera from the external body surface.
ii. Fresh, sterile surgical instruments (scissors, forceps and scalpels) were used for cutting the skin, for cutting the muscular abdominal wall, and for each site sampled for bacteriology.

iii. A new pair of clean latex gloves, worn over thicker protective rubber gloves, were used at each stage in the process of opening body cavities or dissection, and at each sampling site.

iv. For each intestinal sample a loop, or in the case of the caecum and rumen a pouch, of the intestine was isolated using two new plastic, disposable food bag clips (Klippits, Lakeland Ltd.) applied side by side across the intestine. The isolated piece of intestine was excised by cutting between the clips, and was then removed immediately for processing. The cut ends of intestine remaining in the abdomen were then sealed within a clean plastic bag, or the inverted latex glove which had been used to hold the cut ends, which was clamped around the intestine using another plastic clip. This process is illustrated in Figure 2-b. The sealed cut ends of the intestine were then released back into the abdomen.

v. Samples were placed immediately on new, clean, labelled plastic or expanded polystyrene trays and passed to an assistant for processing for histological and bacteriological examination Cross-contamination was avoided by using separate instruments for each sample.

2.6.3 Animal procedures

2.6.3.1 Oral inoculation experiments

A. Neonatal lambs

The lambs had pre-inoculation faeces samples taken at four days of age which were cultured for *E. coli* O157 by the IMS procedure (Section 2.2.9).

Crossbred lambs were allowed to suckle immediately after parturition and received milk replacer by bottle over the ensuing five days. At six days of age, animals were each inoculated orally by syringe with a four-strain mixture of *E. coli* O157:H7. Animals were observed at intervals of four hours. Rectal temperatures were determined at the time of inoculation, at 15 hours p.i. and at 24-hour intervals thereafter. Rectal swabs were taken from these lambs at four days of age, prior to inoculation, and also at 6 and 12 hours p.i., and thereafter approximately 1 g of faeces were obtained from lambs alive at 36, 60 and 84 hours p.i. The faeces samples were either freshly-voided or taken directly from the rectum by digital retrieval using a clean glove.

Tissues were sampled under terminal anaesthesia from the lambs at time points between 12 and 84 hours p.i. On each occasion, the lamb was anaesthetised with a 200 mg/ml pentobarbitone euthanasia preparation given intravenously to effect. When unconscious, the lamb was placed in left lateral recumbency and the right flank and inguinal area was soaked

with a chlorhexidine-based surgical scrub (Hibiscrub, SSL). A disposable clean surgical drape with a 10 x 10 cm hole in the middle was placed over the right flank and secured with sterile towel clips. U-shaped incisions were made in the skin and then in the underlying abdominal musculature, starting from the dorsocaudal quadrant of the flank and coursing in succession ventrally, cranially and dorsally. The cutaneous and muscular flaps so created were reflected dorsally and secured with a sterile towel clip. The caecum, lying across the exposed abdominal viscera, was grasped and the ileocaecal junction was located. Samples were taken using the clean technique (Section 2.6.2) in the following order: ileum, jejunum, duodenum, tip of caecum, proximal loop of ascending colon ('Colon 1'), spiral colon (junction of centripetal and centrifugal parts, 'Colon 2'), terminal colon ('Colon 3') and mesenteric lymph nodes. The lamb was then euthanased with an intracardiac injection of pentobarbitone and placed in dorsal recumbency. The skin was reflected bilaterally from the inguinal area and, using fresh instruments, the pubic symphysis was cut with a scalpel and the pelvic cavity plus retroperitoneal tissues were exposed. The rectum was sectioned transversely immediately cranial to the anus and at the cranial limit of the pelvic cavity, and removed for examination. The submandibular tissues and tongue similarly were dissected and reflected to expose the pharynx for excision of the tonsils. Finally the rumen wall and pieces of liver, kidney and spleen were sampled. The sites of intestinal samples are shown in Figure 2-c.

B. Six- to11-week old lambs

Crossbred animals from the VLA Weybridge or University of Bristol Langford flocks, were faeces sampled on one (Langford flock) or two (Weybridge flock) occasions. The faeces samples were subjected to the *E. coli* O157 IMS and culture procedure (Section 2.2.9). The animals were then housed in groups of four in the Aerobiology Unit containment facility at Langford, and were sheared on the right flank and given one week to adjust to the new environment and a complete pelleted diet (Ewbol Lambwena, BOCM) with water, provided *ad libitum*. Each animal was then inoculated orally by syringe with *E. coli* O157:H7. Each group was inoculated either with the four-strain mixture or with a single strain. Animals were observed twice a day and faeces samples were collected daily from each animal for bacteriological analysis, either by digital retrieval from the rectum or by collection of freshly-voided faeces. One animal was anaesthetised with intravenous pentobarbitone, tissue-sampled and euthanased as described in Section 2.6.3.1A, on each sampling day. Following aseptic preparation of the right flank, samples were collected and euthanasia was performed as described for the neonatal lambs. For these older animals the pelvic symphysis was cut using a small, sterile hacksaw following cutting and reflection of the skin using a scalpel and forceps. At this stage it was possible to pull apart by hand the

two halves of the pelvis, exposing the pelvic cavity. In one group of animals, mesenteric lymph nodes and tonsils were not sampled.

Two uninoculated ten week-old animals (Weybridge flock) and two six-week old animals (Langford flock) were selected at random, and tissues were sampled under terminal anaesthesia, as described for the inoculated animals.

C. Six-month old lambs

Ten six-month old Merino-cross lambs from the VLA Weybridge flock were faeces-sampled on two occasions and the samples subjected to *E. coli* O157 IMS and culture (Section 2.2.9). Two lambs were selected at random and were euthanased by intravenous injection of a proprietary pentobarbitone euthanasia preparation. Tissue sampling, as described in Section 2.6.3.1A but omitting the pelvic splitting and rectal samples, was performed rapidly, using the clean technique described in Section 2.6.2. The remaining eight animals were inoculated orally with the four-strain *E. coli* O157:H7 mixture by calibrated drenching gun. The lambs were housed in pairs and were observed twice daily. Rectal temperatures were taken daily from all animals. One pair was euthanased and tissues were sampled similarly to the uninoculated animals at each of four time points. Since two animals were sampled on each occasion, samples from the second animal were homogenised in PBS containing 15 % glycerol for snap freezing followed by slow thawing, IMS and culture on a subsequent day.

2.6.3.2 Intestinal loop experiments

Four crossbred lambs, of four to six months of age, were used. Faeces were collected from all animals on three occasions between birth and surgery. The faecal samples were cultured selectively for *E. coli* O157 using IMS as described in Section 2.2.9. After shearing the ventral abdomen and overnight feed withdrawal, each animal was premedicated with 0.2 mg/kg xylazine (Rompun 2 %, Bayer) given by deep intramuscular injection, and then anaesthetised using a combination of diazepam (Valium, Roche) and ketamine (Ketaset, Fort Dodge) given intravenously to effect. Following intubation, anaesthesia was maintained by inhalation using isoflurane (Isoflurane RM, Merial) in oxygen via a circle circuit. During anaesthesia, lactated Ringer's solution (Animalcare) was administered by intravenous infusion at 5-10 ml/kg/hour. Anaesthetic monitoring included the measurement of rectal temperature, arterial blood pressure, pO_2 and pCO_2 in the anaesthetic circuit, and an electrocardiograph. Using an aseptic surgical technique, a ventral midline laparotomy was performed and the spiral colon was exteriorised. Ligated intestinal loops, each approximately 10 cm long and separated by a short intervening segment, were created by tying pairs of

encircling braided nylon ligatures around the spiral colon, preserving the mesenteric blood supply to each segment. A 2 ml volume of inoculum containing a single bacterial strain (Section 2.2.6) was then introduced into alternate loops by transmural injection using a 25-gauge needle. Intervening loops served as controls with inoculation of 2 ml of the sterile carrier medium (PBS or DMEM, according to that used in the bacterial inocula) alone. The injection site in each loop was sealed on the serosal surface by thermocautery.

Following inoculation, the intestine was replaced in the abdomen and the laparotomy incision was closed. The animal was maintained under anaesthesia in dorsolateral recumbency, with venting of rumen gas via a paracostal rumenostomy. Six hours after inoculation the ligated intestinal segments were re-exteriorised and excised for fixation. The animal was then euthanased, without regaining consciousness, using intravenous pentobarbitone.

2.7 Pathological techniques

2.7.1 Fixation

Tissues were fixed by immersion in 10 % neutral buffered formalin (Appendix 1) at room temperature for at least 24 hours. Tubular pieces of intestine were opened longitudinally with blunt-ended scissors for about half their length before immersion, except for excised ligated colon loops which were opened along their whole length. All intestinal tissues were placed into labelled individual pots of formalin and were gently dipped several times in the fixative, to dislodge loosely adherent intestinal contents, before being left to fix. The time between death or excision and immersion in fixative was considered to be of prime importance due to the rapidity of intestinal mucosal autolysis, and efforts were made to observe the length of this period on a representative series of occasions.

For some experiments, small samples of intestinal tissues were also fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4) at room temperature, potentially for processing for electron microscopy.

2.7.2 Histopathological studies

Formalin-fixed intestinal tissues were trimmed into strips and embedded in paraffin wax using an automatic processor (Citadel 2000, Shandon) on a long (3-day) cycle. In one experiment, glutaraldehyde-fixed tissues were processed similarly. One long edge of each tissue strip was orientated towards the cutting face of the wax block, such that conventionally-orientated sections of the intestinal wall including all layers and perpendicular to the plane of the mucosal surface, were obtained. Additionally, some large

intestine sections in some experiments were embedded with the mucosa presented parallel to the sectioning face. Two or more sections were prepared from each anatomical sampling site, as detailed in chapters 4, 5 and 6. In some cases multiple sections were prepared by cutting at different levels from the same wax block. Representative pieces of liver, kidney, spleen (and of tonsil and mesenteric lymph nodes where collected) were also embedded and sectioned. Sections were cut at 4 μm on a sledge microtome (Leitz), placed onto glass microscope slides coated with glycerin-albumen (R. A. Lamb), dried, and baked on a 60 °C hotplate for 20-30 min to ensure adequate adhesion. The sections were then dewaxed in a clearing agent (Histo-Clear, National Diagnostics) for at least 6 min and stained with haematoxylin and eosin (H&E) by hand according to the protocol in Appendix 2.

Intestine sections were examined, and remarks recorded, for several features:

- Tissue preservation, assessed mainly by the condition of the mucosa including the degree of any epithelial detachment.
- Villus morphology, including the villus:crypt length ratio, in the small intestine. Villus:crypt ratios representative of the section overall were estimated by eye.
- Cell population and overall cellularity of the lamina propria.
- Density and type of leucocytes within the mucosal epithelium.
- Presence and nature of adherent organisms or luminal contents at the mucosal surface.

In addition, other features were noted and recorded when it was considered to be relevant. Generally, one section from each anatomical site was examined in detail using the above assessment criteria, and subsequent sections from the same site were surveyed in detail only for features of the epithelial mucosa, such as attached bacteria.

When appropriate, to characterise or clarify bacterial features, additional sections were cut and were stained by Gram-Twort, Giemsa or Warthin-Starry methods (Appendix 2).

2.7.3 Immunohistochemical studies

Wax-embedded 4 μm sections of tissue were prepared from the same blocks as the H&E-stained sections, for immunoperoxidase (ipx) immunohistochemistry. Tissues were selected for this procedure either on the basis of features noted on H&E-stained sections, or as a comparative technique to H&E-stained sections for screening (intestinal loop studies), in which case similar numbers of H&E- and ipx-stained sections were prepared.

To maximise adhesion, tissue sections for ipx were floated onto glass slides which had been coated with the adhesive 3-aminopropyltriethoxy-silane (organo-silane, Sigma). Slides bearing sections were then baked on a hotplate at 60 °C for 20-30 min and held overnight in

a 37 °C oven. Immunostaining was performed using the peroxidase-antiperoxidase (PAP) complex method (Sternberger et al. 1970; Appendix 2). The primary antibodies used were polyclonal rabbit antisera specific for a particular *E. coli* 'O' antigen, produced by the VLA Weybridge *E. coli* typing unit by inoculating rabbits with heat-treated *E. coli* preparations, and intended for use in somatic antigen typing of cultures. Working dilutions of between 1:500 and 1:4000, depending on individual antisera, were found to be optimal when used on control positive sections.

The staining process was verified using the test antiserum on a positive control section, and the lack of non-specific staining of organisms by the primary antiserum was demonstrated using the test antiserum on a negative control section containing *E. coli* organisms of an alternative serogroup. The absence of non-specific staining by any other element in the process was demonstrated by using, for each tissue block, a section stained with an unrelated primary antiserum. This control antiserum was either a VLA rabbit polyclonal antiserum for another serogroup, diluted to its normal working immunostaining strength, or normal rabbit serum (Sigma) diluted 1:1000 in PBS.

The positive control material comprised tissue sections of calf intestine from a natural clinical case of *E. coli* O26 enteritis, an agar-embedded, cultured *E. coli* O26 strain and an agar-embedded, cultured *E. coli* O157:H7 strain. No intestinal material containing *E. coli* O157:H7 organisms at a sufficiently high density for reliable control material was available. The agar-embedded preparations were generated from an overnight broth culture of the appropriate serogroup which had been fixed by mixing with an equal volume of 3 % neutral buffered formalin. The fixed suspension in a 1.5 ml microcentrifuge tube (Eppendorf) was sedimented by centrifugation at 13 000 rpm (Biofuge 13, Heraeus Sepatech) for 3 min and the supernatant was removed. A 1 % solution of high strength agar (Agar powder - high gel strength, BDH Ltd.) in distilled water was melted in a microwave oven, the microcentrifuge tube was filled with the molten agar when it was at approximately 60 °C and the bacteria were resuspended using a pipette. The tube was accelerated to 13 000 rpm for 30 seconds to concentrate the bacteria at the bottom of the tube as the agar set. When cooled, the agar plug was removed from the tube using a mounted needle and the tapering cone-shaped tip containing the concentrated bacteria was cut off. It was bisected along its long axis and each half was wax-embedded routinely, with the flat cut face presented at the sectioning face of the wax block. When sectioned at 4 μm, a roughly triangular sample was obtained, with bacteria concentrated at the apex and along the two adjacent sides, and with an even, lower-density distribution of bacteria present throughout the rest of the section.

2.7.4 Electron microscopy

2.7.4.1 Scanning electron microscopy

SEM of infected cell monolayers was performed on glutaraldehyde-fixed specimens by the VLA Weybridge Electron Microscopy Unit. Monolayers on coverslips were fixed for 16 hours in 3 % (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, washed in phosphate buffer and post fixed in 1 % (w/v) osmium tetroxide in the same buffer. Specimens were rinsed in six changes of phosphate buffer, dehydrated in ethanol, immersed in acetone and subjected to critical point drying with liquid CO_2. Dried specimens were fixed to aluminium stubs with conductive silver paint, sputter coated with gold and examined using a Cambridge Stereoscan S250 Mark III scanning EM at 10-20 kV. Scanning electron micrographs were recorded as black and white photographic prints.

2.7.4.2 Transmission electron microscopy

A. Wax block retrieval of tissue

The technique was described by Skuse (2002a). Features suitable for TEM were identified using light microscopy (LM). By comparing the glass slide section with its corresponding tissue block, the area on the cutting face of the block most likely to contain a part of the feature seen in the section was identified and marked. Using a mounted razor blade, a pyramidal piece of wax and tissue was carefully excavated from the face of the block, with the base of the pyramid comprising the cutting face of the block in the area of interest and being approximately 2 mm square (Figure 2-d). This excised tissue was then dewaxed, hydrated, post-fixed in osmium tetroxide, dehydrated and embedded in epoxy resin, according to the protocol in Appendix 2. Great care was paid to the orientation of the tissue in the resin embedding capsule, in order to get the face taken from the cutting surface of the wax block again at the cutting surface of the resin block.

Once embedded, 1 μm semi-thin sections were cut using an Omu3 ultramicrotome (Reichert), stained with 1 % toluidine blue in 1 % borax solution for 1 min on a hotplate at 80 °C, and examined under the LM. If features likely to be of interest were seen, thin (approximately 90 nm) sections were prepared, mounted on thin-bar 300 mesh copper grids and stained with saturated methanolic uranyl acetate for 10 min, then with Reynold's lead citrate for 5 min, according to the protocols in Appendix 2. The sections were examined in a Philips 201 transmission electron microscope. Photomicrographs were taken of features of interest.

B. Retrieval of tissue from light microscope sections

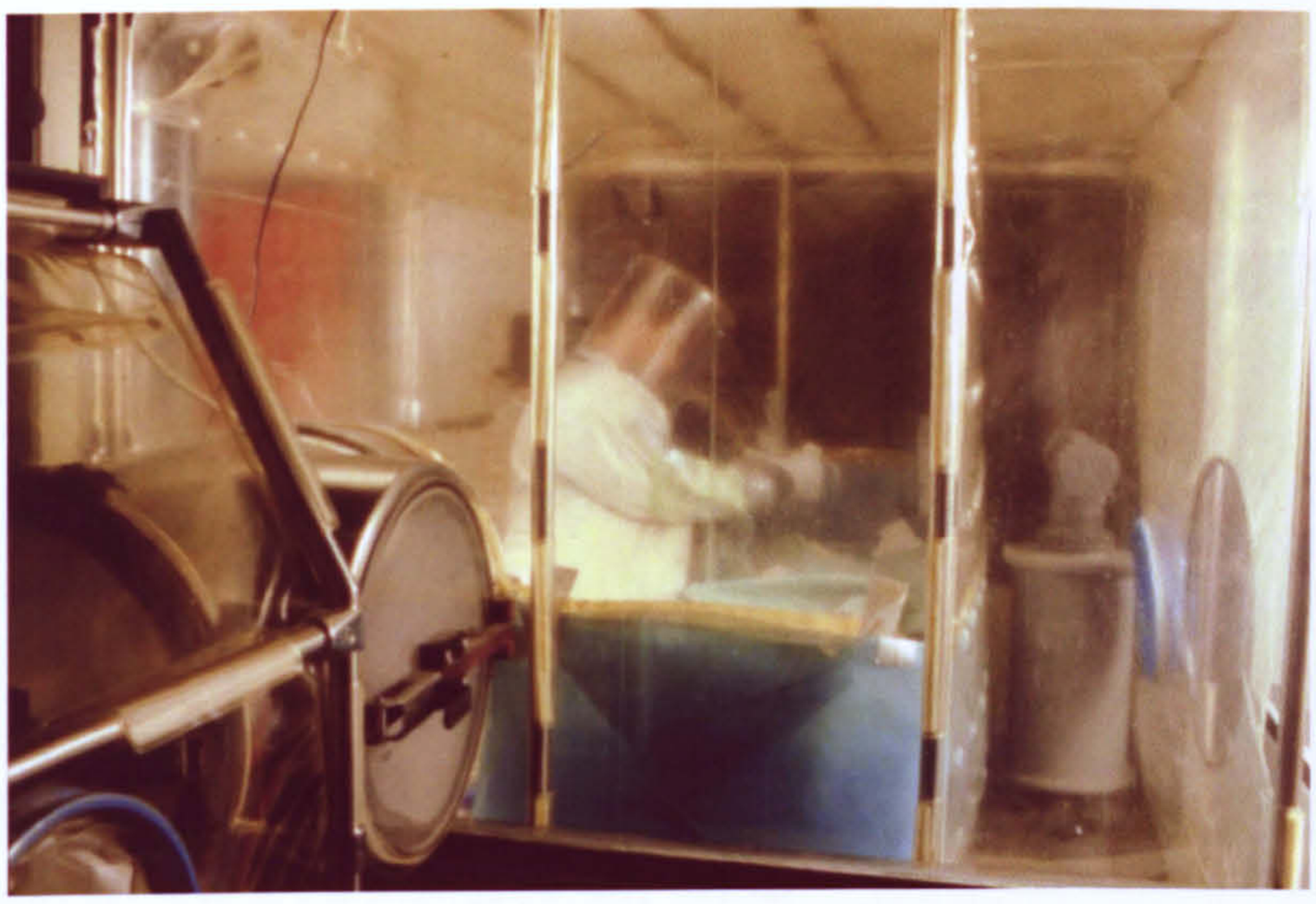
When the lesion of interest was too small to be found in tissue removed from the wax block, an alternative technique was used (Skuse 2002b; Appendix 7), adapted from previous reports (Glauert 1975; Bretschneider et al. 1981). The area of interest was ringed on the reverse of the slide with a diamond tipped pen. The coverslip was removed by soaking the slide in Histo-Clear until the DPX mountant was loosened, and the slide was then soaked in Histo-Clear for a further few hours. The slide was placed in 100 % alcohol for two or three changes of 30-60 min each, and then placed in a sealed container of acrylic resin (LR White resin, London Resin Company) for two or three changes of at least 12 hours each. One plastic EM processing capsule was completely filled with liquid resin with added polymerisation accelerator, so that the meniscus was convex. The slide was removed from the resin container and as much excess resin as possible was drained off. The slide was placed section side down on top of the resin-filled capsule with the marked area of interest in the centre of the resin, and the resin was allowed to polymerise over several minutes. The reverse of the slide was then sprayed liberally with freezing spray (Cool-Jet, R. A. Lamb Ltd.). When a crack was heard, indicating partial separation of the resin from the slide, the capsule, including an embedded piece of the tissue section, was snapped away from the slide by hand. After removal of the resin block from the capsule, the cutting face was trimmed to permit thin sections of the area of interest to be cut.

With this technique, only a 4 μm thickness of tissue, well-orientated at the cutting face of the resin block and very probably containing the feature of interest, was available for thin sectioning, and so semi-thin sections were not prepared as a preliminary step. Thin sections were cut, with extreme care being exercised in respect of the orientation of the face of the block to the knife. This was done immediately after detachment of the tissue from the glass slide, because otherwise slight contraction of the embedding resin led to concavity of the cutting face of the block.

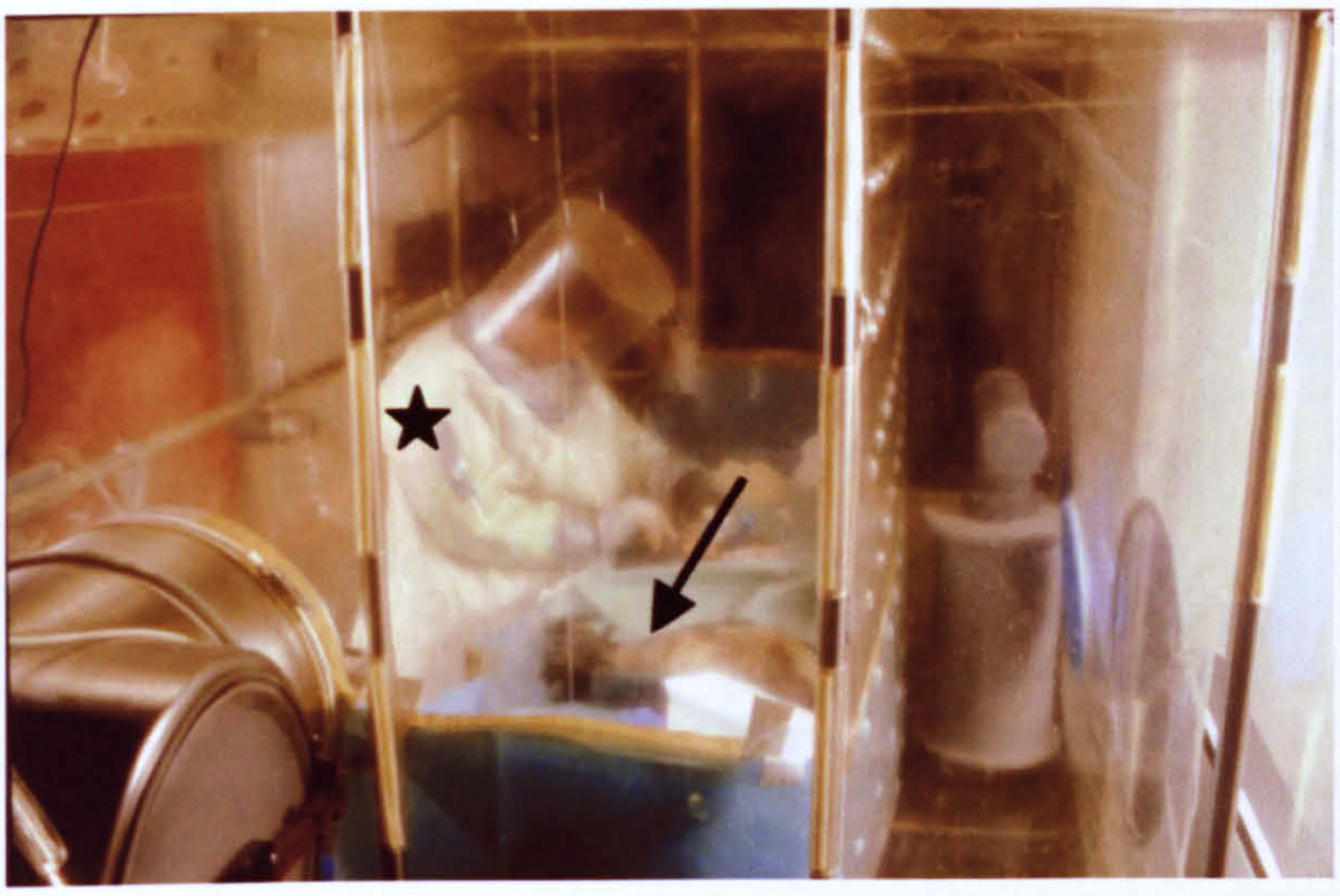
2.7.4.3 Immuno-electron microscopy

Immunogold labelling (Faulk and Taylor 1971) of bacteria in some TEM sections was performed, using the same primary O157 antiserum that was used for ipx staining (Section 2.7.3). A two-layer staining technique, using a gold-labelled anti-rabbit secondary antibody, was performed (Skuse 2002c; Appendix 2) using a high concentration (1:5 dilution) of primary antibody. A negative control procedure, using either normal rabbit serum (Sigma) in place of the primary antibody, or omitting the primary antibody, was performed in parallel.

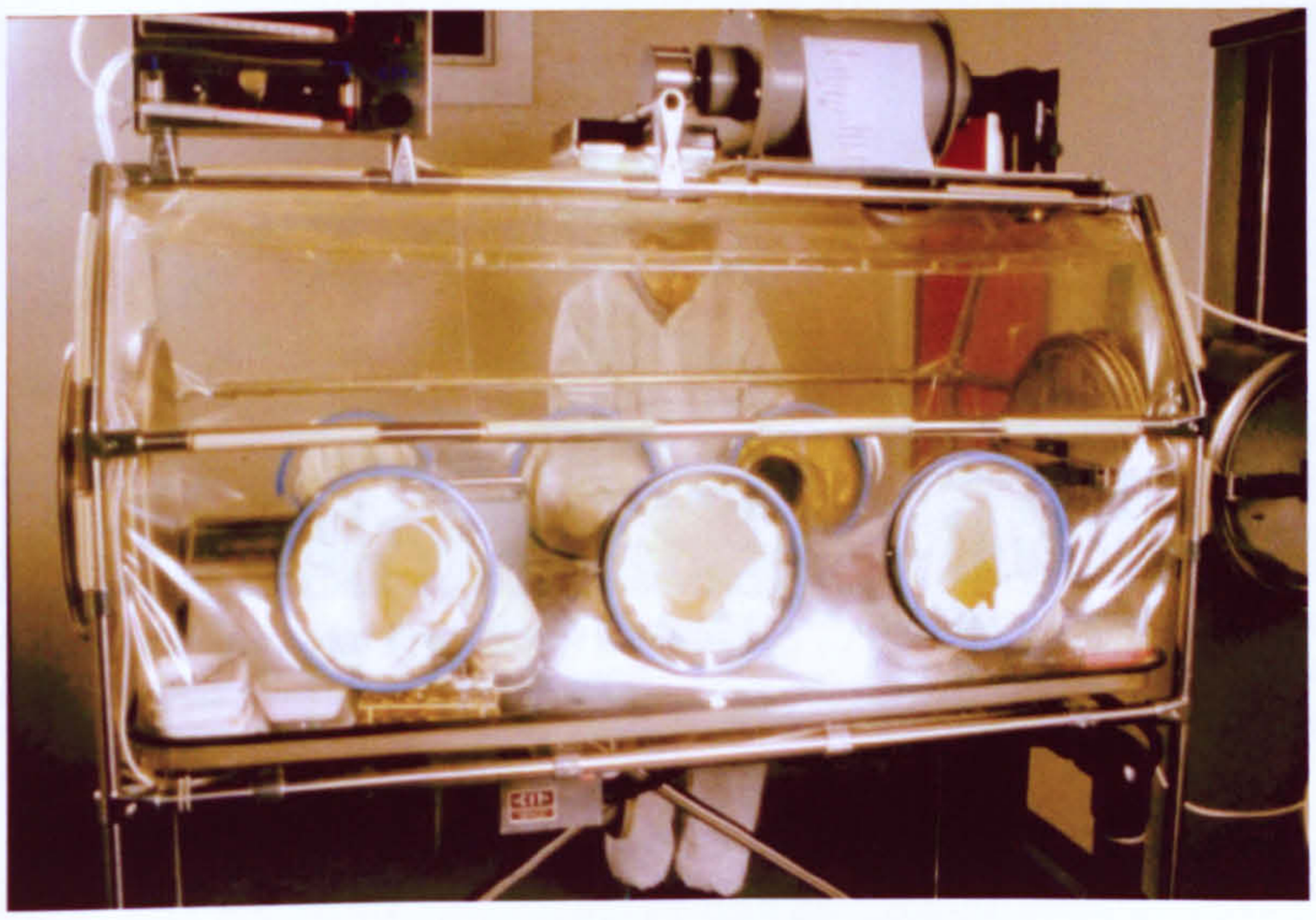
Figure 2-a: Aerobiology Unit category 3 containment animal facility



i. The operating chamber (middle) is linked via hatches to the animal room (background, darkened) and the workstation (foreground, left) The airspace in the animal room connects only with the internal airspace of the rest of the apparatus.

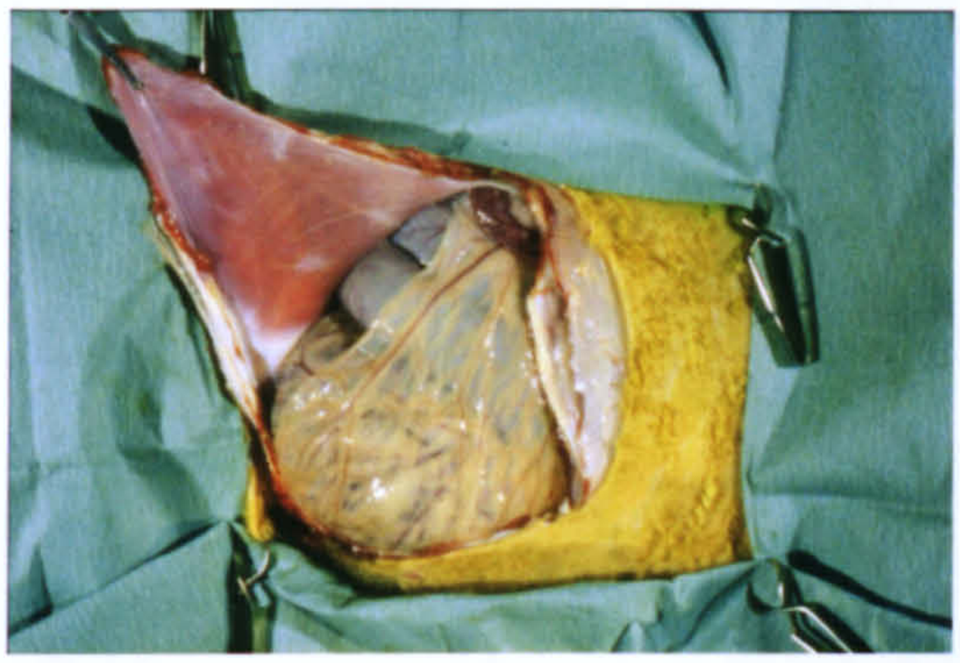


ii. Operating chamber. The half-suit is sealed to the edge of a horizontal aperture at waist level, thus the operator (starred) can stand in the aperture and work on the animal (arrowed) whilst remaining in the external airspace.



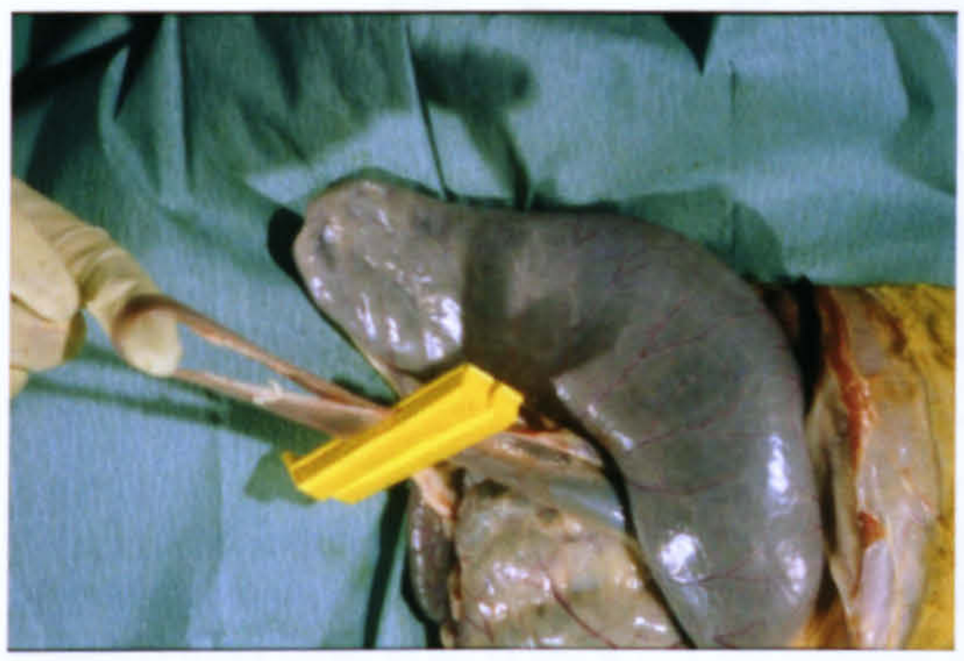
iii. Workstation. Material passed through the hatch on the right is manipulated using integral sleeves and gloves.

Figure 2-b: Clean sampling technique demonstrated on a dead animal

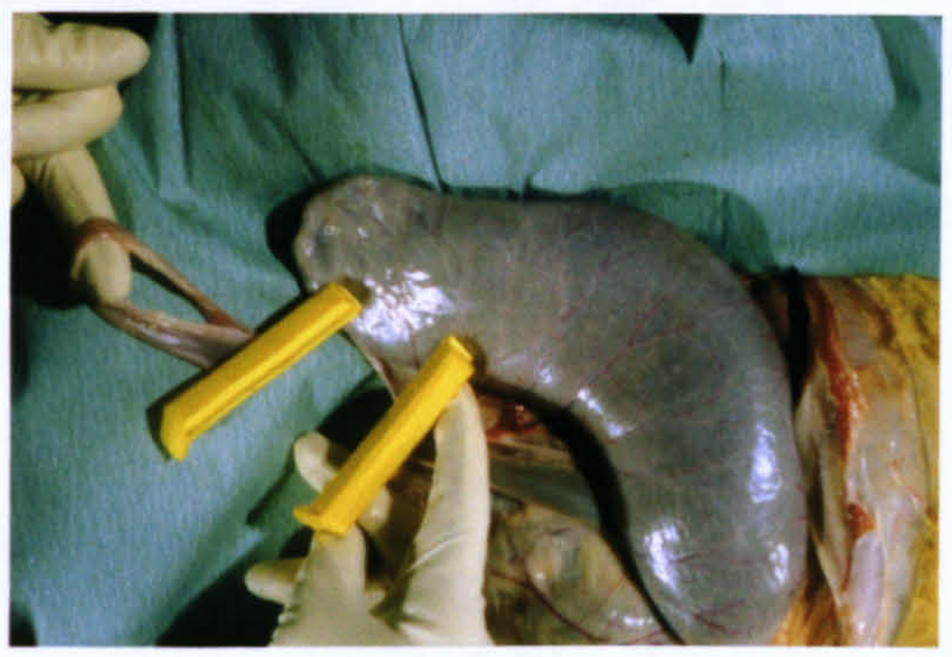


i. After disinfecting and draping, the body wall on the right flank is opened in two layers.

ii. The caecum is located and exteriorised.

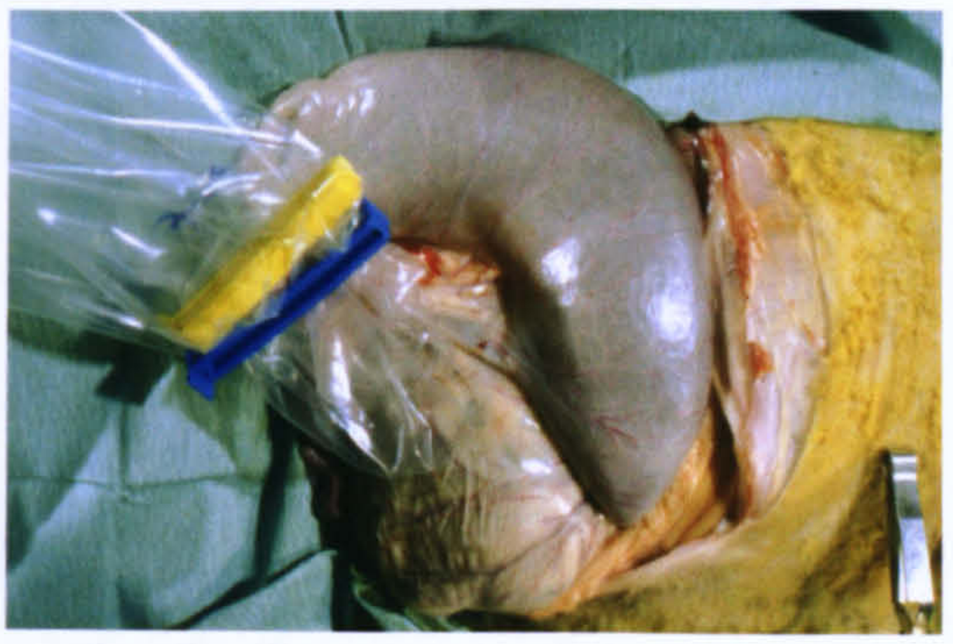


iii. The duodenum is located, a loop is freed from its mesentery and double-clipped.



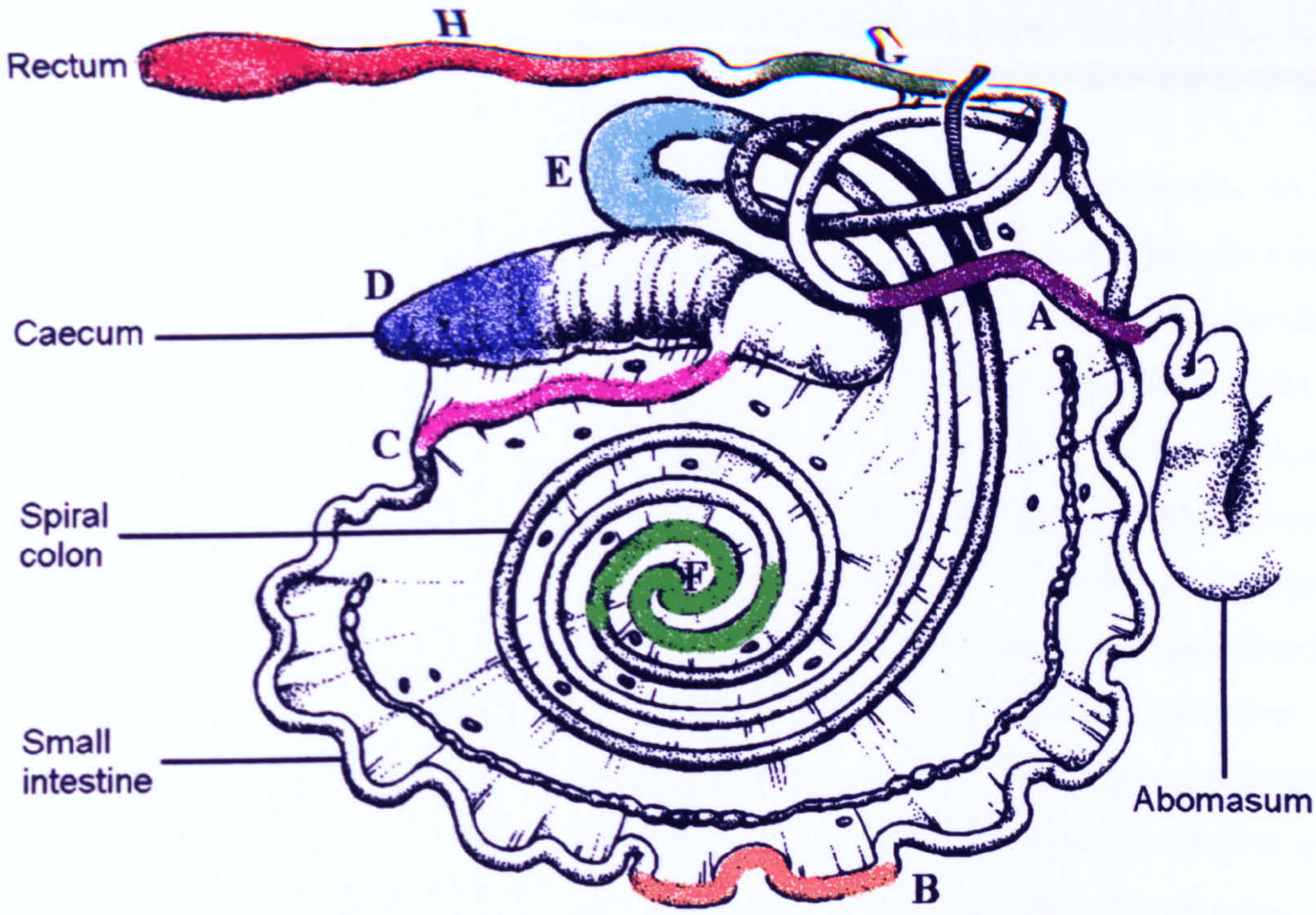
iv. The intestinal loop is cut between the clips and processed immediately. The remaining cut ends are carefully held to avoid contamination.

v. A clean plastic bag (or latex glove) is placed carefully over the clip-sealed cut ends



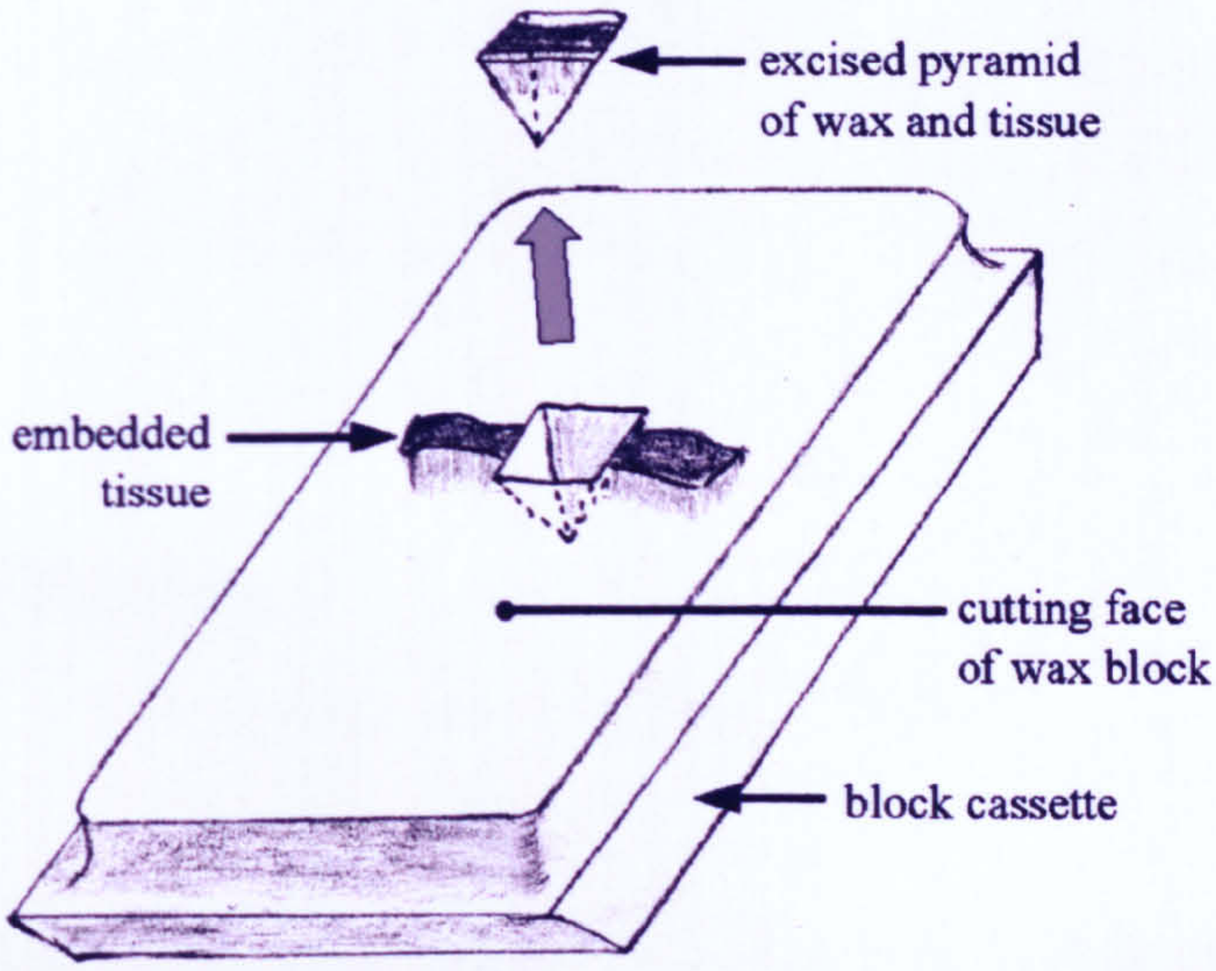
vi. The cut ends and clip are sealed within the bag using another clip (blue) before being released and the process repeated with the next sample

Figure 2-c: Schematic diagram of intestinal sampling sites



Key:
A (purple) duodenum; **B** (orange) jejunum; **C** (pink) ileum; **D** (blue) caecum; **E** (cyan) proximal loop of ascending colon; **F** (bright green) spiral colon; **G** (dark green) terminal colon; **H** (red) rectum.
Adapted from Dyce et al. (1987).

Figure 2-d: Technique of tissue recovery from wax block for electron microscopy



Chapter 3 – *IN VITRO* STUDIES

3.1 Introduction

In order to be confident that the test strains of *E. coli* O157:H7 used in the present studies were comparable to those in the field, it was decided that appropriate human and animal sources for strains should be used, and that screening of several candidate strains for known and putative virulence and persistence factors should be performed. Thus, it was intended that test strains were recently derived from relevant natural environments, possessed the normal complement of factors of 'typical' EHEC O157:H7, as defined by Nataro and Kaper (1998), and reflected some of the diversity, in terms of Shiga toxin genes, acid tolerance and sources, to be found in *E. coli* O157:H7 strains. Four *E. coli* O157:H7 strains were selected for use in *in vivo* studies on the basis of certain genotypic and phenotypic features (Section 3.6), and on the ability to generate spontaneous antibiotic resistance marked derivatives which would grow acceptably in mixed culture (sections 3.4 and 3.5.4), which would enable their retrieval, identification and enumeration after inoculation of animals. The four *E. coli* O157:H7 strains and their antibiotic resistance marked derivatives selected for *in vivo* studies were subjected to further phenotypic tests in an attempt to discern correlations between strain behaviour *in vitro* (present chapter) and *in vivo* (chapters 4, 5 and 6). These subsequent *in vitro* studies also included two strains not included in the original characterisations: an *E. coli* O26:H11 (EC1537), used as a control strain in the intestinal loop studies (Chapter 4), and a non Shiga toxin-producing *E. coli* O157:H7 (NCTC 12900), used in a later *in vivo* experiment (Experiment 6/4, Chapter 6). In addition, characterisation of a naturally-occurring bovine AEEC (Strain 85440, Section 2.2.1.2) was undertaken, in view of its potential role as a control organism. The control strains used (EPEC B171, bovine EPEC 85440, bovine EHEC-like EC1537 and *E. coli* K12 DH5α) are described in Section 2.2.1.2.

3.2 Strains examined

The *E. coli* O157 strains examined are summarised in Table 3-A.

3.3 Genotypic characterisation of experimental strains

PCR was used to detect genes using specific primer pairs and template DNA extracted from the strain under examination (Section 2.3). The presence of the gene was inferred if a PCR product of the expected size was seen, using agarose gel electrophoresis and ethidium

bromide staining, and if the negative control reaction, lacking template DNA, yielded no product. For the *E. coli* O157:H7 strains, the chromosomal genes of the two Shiga toxin types (*stx1* and *stx2*) plus the LEE gene *eaeA* encoding intimin were amplified together in a multiplex PCR (Cookson et al. 2002a). The strains were also examined for the presence of the virulence plasmid genes *katP*, *espP* and *etpD* plus the EHEC-*hly* gene cluster, encoding respectively: catalase peroxidase, serine protease, part of a putative type II secretion system and enterohaemolysin (Section 1.4.2.2E). In addition, amplification of the *rpoS* gene, encoding the RpoS stationary-phase RNA polymerase sigma factor (Section 1.4.2.2C) was performed. A functional *rpos* gene is associated with expression of virulence factors in *E. coli* (Loewen and Hengge-Aronis 1994), acid resistance in *E. coli* O157:H7 (Cheville et al. 1996) and a high level of excretion of *E. coli* O157:H7 from inoculated calves (Price et al. 2000).

Table 3-A: *E. coli* O157:H7 strains examined *in vitro*

Strain	Source	Supplier	Clinical features	*stx* genes	Phage type
131567	Human	LEP	Not stated	1	8
134107	Human	LEP	Diarrhoea	1	8
138345	Human	LEP	Diarrhoea	1 + 2	2
139229	Human	LEP	Bloody diarrhoea	1 + 2	4
139579	Human	LEP	Diarrhoea, abdominal pain	2	4
139969	Human	LEP	Not stated	2	21/28
140019/0	Human	LEP	Diarrhoea	1 + 2	14
140065	Human	LEP	Asymptomatic in-contact of symptomatic child	2	2
NCTC 12900	Human	NCTC	Human diarrhoea (Willshaw et al. 1994)	–	54
EC157	Bovine	VLA	Traced isolate with identical PFGE pattern to fatal HUS strain.	1 + 2	1
EC218	Bovine	VLA	-	1 + 2	54
EC222	Bovine	VLA	-	2	32

LEP: Laboratory of Enteric Pathogens, Central Public Health Laboratory.
NCTC: National Collection of type Cultures

For Strain 85440 the genes *eaeA* and *bfpA* were sought, the latter encoding the BFP of typical human EPEC (Section 1.1.3.1). This strain was reportedly non Shiga toxin-producing on Vero cells (Pearson et al. 1989), therefore Shiga toxin genes were not looked for.

The results from all strains are summarised in Table 3-B and typical ethidium bromide-stained gel electrophoresis images are shown in Figure 3-a. All gene products were of the expected molecular mass. It can be seen that one *E. coli* O157:H7 strain (138345) lacked two

of the four virulence plasmid (pO157) genes, suggesting either that the genes involved had been lost from the plasmid or had lesions at the primer binding sites, rather than the genes being lost due to loss of the plasmid from the strain. The Shiga toxin PCR reactions were in agreement with those reported for the strains from the LEP. The 85440 strain, being non Shiga toxin-producing, possessing the LEE gene tested for and lacking the *bfpA* gene, genotypically resembled an atypical human EPEC.

Table 3-B: Summary of genotypic strain characterisation

	Strain	*stx*	*eaeA*	*hlyA-C*	*katP*	*espP*	*etpD*	*rpoS*	*bfpA*
E. coli O157:H7	131567	1	+	+	+	+	+	+	
	134107	1	+	+	+	+	+	+	
	138345	1 & 2	+	–	+	–	+	+	
	139229	1 & 2	+	+	+	+	+	+	
	139579	2	+	+	+	+	+	+	
	139969	2	+	+	+	+	+	+	
	140019/0	1 & 2	+	+	+	+	+	+	
	140065	2	+	+	+	+	+	+	
	EC157	1 & 2	+	+	+	+	+	+	
	EC218	1 & 2	+	+	+	+	+	+	
	EC222	2	+	+	+	+	+	+	
	85440		+						–

Shaded cells indicate that the PCR test was not performed for that strain/gene combination.

3.4 Generation of spontaneous antibiotic resistant mutants

Antibiotic resistant strains were generated as described in Section 2.4. The following were made: EC218 rif^r (rifampicin resistant), EC222 nal^r (nalidixic acid resistant), 139579 $spec^r$ (spectinomycin resistant) and EC157 str^r (streptomycin resistant). However, Strain 139579 $spec^r$ failed to grow acceptably in mixed culture (Section 3.5.4), with a one log_{10} unit lower TVC achieved by this strain than by other strains in the mixture. A $spec^r$ mutant of an alternative strain (140065) had an untypical, small colonial morphology, therefore Strain 139579 $spec^r$ was substituted with Strain 140065 nal^r and a double-marked EC222 $nal^r rif^r$ mutant was generated, which grew acceptably (no marked growth arrest and a final TVC within one log_{10} unit of the other strains) both alone and in mixed culture (Section 3.5.4). The final set of strains used in the mixed inoculum (Chapters 5 and 6) therefore was: 218 rif^r, 222 $nal^r rif^r$, 140065 nal^r, and EC157 str^r.

3.5 Phenotypic characterisation of experimental strains

3.5.1 Protein expression and secretion

A. Initial strain characterisation

Initially, all candidate *E. coli* O157:H7 strains were screened for intimin expression by Western blotting using cultures grown in DMEM (sections 2.5.4.2A and 2.5.4.3B). Growth of bacteria in DMEM promotes the expression of LEE proteins in EPEC (Knutton et al. 1997) and EHEC (Ogierman et al. 2000; Abe et al. 2002). The results are shown in Figure 3-b. All strains tested expressed intimin of the expected molecular weight (94-97 kDa Jerse and Kaper 1991; Louie et al. 1993), which was detected by the polyclonal γ-intimin antibody as shown in Figure 3-b. However, as shown, there were differences between the amounts present for each strain under the conditions used.

B. Comparison between parent and antibiotic resistance marked strains

After DMEM induction (Section 2.5.4.1A), comparison between the parent and antibiotic resistance marked derivative strains was made of: protein secretion (Section 2.5.4.3A), EspA secretion (Section 2.5.4.3B) and intimin expression (Section 2.5.4.3B). Procedures were performed in duplicate, and representative silver-stained SDS-PAGE gels and Western blot membranes are shown in Figure 3-c. It was attempted to standardise the TVC of the PBS or DMEM bacterial suspensions used for each preparation, and the measured TVC was recorded alongside gel and membrane images. This permitted an approximate comparative assessment of the amount of protein of interest per cfu when gels and Western blots were evaluated. It can be seen that, with the possible exception of strains 222/222 $nal^r rif^r$, where secreted protein levels were low in both cases, antibiotic resistance marking was associated with reduced secretion of proteins under DMEM induction. The apparent molecular mass of the EspA (approximately 20 kDa) is lower than the 22-25 kDa previously shown for EspA of *E. coli* O157:H7 and O26:H- (Ebel et al. 1998), although distortion of bands at the edge of the gel and imprecision in the marking of bands on the membrane may account for this. The double bands of similar apparent molecular mass which are labelled by the EspA antiserum have been reported by other workers (Ebel et al. 1996; Ebel et al. 1998), but the reason for this appearance is undetermined. The possibility that this represents partial disruption of disulphide bonds, resulting in molecules of differing conformation and migration characteristics, is discounted by the fact that the published EspA sequence for *E. coli* O157:H7 (Various 1998) lacks cysteine residues, and therefore would not contain disulphide bonds.

C. Comparison between E. coli O157:H7 and O26:H11 strains

Comparison of protein secretion, EspA secretion and intimin expression between E. *coli* O157:H7 and *E. coli* O26:H11 strains was made, following either one hour's exposure to PBS (Section 2.5.4.1B) or five hours' exposure to DMEM (Section 2.5.4.1A). These preparations simulated the pre-inoculation conditions used for the same strains in the ligated colon loop experiments (Chapter 4).

Incubation in DMEM or PBS and then the concentration, electrophoretic separation and detection of secreted proteins was carried out, in duplicate, as described in sections 2.5.4.1, 2.5.4.3A and 2.5.4.3B. The total viable counts, plus representative silver- and Coomassie-stained SDS-PAGE gels and EspA-probed Western blots resulting from one- and five-hour incubations in PBS and DMEM respectively are shown in Figure 3-d. The overall secretion of proteins, as detected by silver staining, showed enhancement with DMEM incubation for all the *E. coli* O157:H7 strains. This included discrete double bands around 20 kDa consistent with EspA (Ebel et al. 1998), and strong bands between 30 and 45 kDa consistent with EspB and EspD (38 and 40 kDa respectively; Kaper et al. 1998; McNally et al. 2001). The EC1537 (O26:H11) strain also showed an overall increase of the secretion of presumed Esp proteins with DMEM on Coomassie-stained gels (Figure 3-d, i), although this was somewhat obscured on silver-stained gels by a relatively high level of other protein bands in the PBS preparation. There was increased secretion of bands in the EspA mass range for all strains, including *E. coli* O26:H11, with DMEM and this was confirmed for EspA by the Western blots.

An assay for intimin expression using incubation of strains in DMEM or PBS, followed by lysis, separation and detection by Western blotting was carried out as described in sections 2.5.4.2 and 2.5.4.3B. The results of a single preparation (Figure 3-e) show the enhancement of intimin expression by a five-hour DMEM incubation when compared with a one-hour PBS incubation, for all *E. coli* O157:H7 and O26:H11 strains tested. The degree of enhancement seen with Strain EC1537 (O26:H11) is uncertain, as the TVC of the DMEM versus PBS preparations differed over a 2.5-fold range.

3.5.2 Association with cultured cells

3.5.2.1 HEp-2 cell line assays

A. Quantitative association assay

Three-hour HEp-2 cell monolayer quantitative association assay (sections 2.5.5.1 and 2.5.5.2) results are shown in Figure 3-f. Each data point represents counts from one well. Association of the AEEC with the HEp-2 cells was at least one $\log_{10}$ unit higher in

comparison with a control *E. coli* K12 (non-AEEC) strain, except for the double-marked EC222 nal^{r}rif^r strain where the count was 8.1 x higher (Figure 3-f, i).

B. Giemsa stained preparations

The combined observations from two preparations (A and B) of Giemsa-stained inoculated HEp-2 cells (sections 2.5.5.1 and 2.5.5.3) are presented in Table 3-C, and Figure 3-g. The strongest localised adhesion (LA) phenotype predictably was demonstrated by the EPEC B171 strain (Section 2.2.1.2). Strain 85440, lacking BFP (Table 3-B), demonstrated less dense and less frequent microcolonies. The wild-type *E. coli* O157:H7 strains all showed the localised adhesion (LA) phenotype, albeit with some variation and with poor expression at the three hour stage. Attenuation of the wild-type LA pattern was seen with all the antibiotic resistance marked *E. coli* O157:H7 strains; at its most marked this attenuation produced small, loose clusters of cells at six hours with Strain EC222 nal^{r}rif^r. Substantial inter-assay variation in the observed adherence pattern was seen in the case of Strain EC222, the difference in the number of inoculated organisms being a possible factor in this. The extent of HEp-2 cell degeneration, as demonstrated by nuclear hyperchromasia and disintegration, was strongly correlated with time post-inoculation, and was much less marked with non-STEC inocula.

Table 3-C: Observations on Giemsa-stained infected HEp-2 monolayers

Strain	Assay	TVC*	3 hour incubation	6 hour incubation
EC218	A	1.3	Small, low density MC, 10 µm, <1/cell, plus areas of more diffuse colonisation.	**Mod dense MC, 15-20 µm, <1/cell. Areas of more diffuse colonisation.**
	B		CC. Loose clusters of 1-7 bacteria on approx. 50 % of cells.	CC, ND common. MC of 15-100 bacteria on 80 % of cells.
EC218 rif	B	0.82	CC. Single or paired bacteria on <10 % of cells.	CC, ND in 30 %. MC of <15 organisms on <10 % of cells.
EC222	A	1.3	2-4 scattered bacteria, <1/cell.	Mod dense MC, 10-15 µm, <1/cell.
	B	0.70	CC. Single and paired bacteria on <1 % cells	CC. Single and paired organisms on approx. 30 % of cells, plus rare MC of <20 organisms.
EC222 nal^{r}rif^r	B	0.62	CC. Single and paired bacteria on 1 % to 10 % of cells.	CC, ND common. Loose clusters, 2 to occasionally 20 bacteria on approx. 50 % of cells.
140065	B	0.65	CC. Adherent bacteria are solitary, paired and occasionally in loose clusters of up to 13, on approx. 20 % of cells.	CC, ND in 20 %. Medium and large (up to 100 bacteria), loose MC on approx. 90 % of cells.
140065 nal^r	B	0.43	CC. Adherent bacteria are solitary, paired and occasionally in loose clusters of up to 13, on approx. 10 % of cells.	CC, ND in 20 %. Bacteria typically scattered singly or paired, on approx. 70 % of cells. Occasional MC of up to 50 bacteria.
EC157	A	1.0	Occasional MC of a few scattered bacteria, <<1/cell.	**Low and mod density MC, 10-15µl, <1/cell.**
	B	0.70	CC. Pairs and occasionally up to 6 bacteria adherent to 1 % to10 % of cells.	CC, ND in 20 %. Scattered bacteria plus MC of up to 50 organisms on 50-70 % of cells.
EC157 str^r	B	0.77	CC. Adherent bacteria on 1 % to 10 % of cells, typically single or paired but occasionally loose MC of up to 15 organisms.	CC, ND in 50 %. Scattered bacteria and MC of typically 10-20 organisms on 30-40 % of cells.
139579	A	0.95	Occasional low-density MC, <1/cell.	Small and dense to large and diffuse MC, 10-20 µm, ≈1/cell.
NCTC 12900	B	0.77	CC. Single bacteria and loose clusters of up to 10 organisms present on 90 % of cells.	CC, minimal/no ND seen. Medium or large, loose MC on 100 % of cells.
B171	A	1.8	**Mod dense MC, 10-15 µm, ≈1/cell.**	**Very dense MC 15-20 µm, >1/cell.**
85440	A	1.2	Low density MC, 10-15 µm, ≥1/cell.	Mod dense MC, 10-20 µm, ≥1/cell.
EC1537	B	0.35	CC. Single bacteria and loose clusters of up to 10 organisms present on 20 % of cells.	CC, ND in 20 %. Adherent bacteria scattered, often in loose MC of up to 50 organisms, on 50 % of cells.
DH5α	B	0.53	CC. Single bacteria and loose clusters, occasionally of 20 or 30 organisms, present on 20 % of cells.	CC, <10 % ND. Adherent bacteria scattered or very loosely clustered. 70 % of cells colonised.
None	B	-	CC. No bacteria seen.	CC, rare ND. No bacteria seen.

* TVC: total viable count of inoculum, x 10^9 cfu. CC: confluent cells. ND: nuclear degeneration. MC: microcolonies. Mod: moderate/moderately. 'Density' refers to the density of bacteria within a microcolony. Size measurements refer to microcolony diameter. **Bold** entries are illustrated in Figure 3-g

C. Fluorescence actin staining (FAS)

Initial characterisation

FAS (sections 2.5.5.1 and 2.5.5.4) was performed to evaluate AE competence *in vitro* on HEp-2 cells. For this, all candidate *E. coli* O157:H7 strains, plus antibiotic resistant derivatives of selected strains, were used. The results are presented in Table 3-D and Figure 3-h. The control EPEC B171 (Section 2.2.1.2) yielded unequivocal FAS-positive lesions by three hours p.i. As previously described (Donnenberg and Nataro 1995), the *E. coli* O157:H7 strains required six hours incubation for the formation of FAS-positive lesions, and the extent and intensity of staining was noticeably less than for the EPEC strain. The intensity of FAS with the *bfpA*-negative bovine EPEC-type strain 85440 appeared to lie between the B171 and *E. coli* O157:H7 strains. Antibiotic resistance marking appeared to have an attenuating effect upon the AE capability of the double resistance marked EC222 $nal^r rif^r$ strain. The use of a control negative organism (EC218 *eaeA* mutant) produced the expected negative result at three and six hours p.i.

Effect of DMEM pre-incubation

A follow-up study was prompted by the observation that antibiotic resistance marking attenuated the FAS capability of strains, possibly to extinction in one case (EC222).

Therefore, the four antibiotic resistance marked component strains of the mixed inoculum (Section 3.6) plus their parent strains were pre-incubated in DMEM for three hours to induce expression of LEE proteins (Ogierman et al. 2000; Goffaux et al. 2001). The cultures were then inoculated onto HEp-2 monolayers, incubated for six hours and FAS was performed (Section 2.5.5.4), in order to compare further the FAS (AE) capability of the parent strains and antibiotic resistance marked derivatives. The results are summarised in Table 3-E.

A comparison between tables 3-D and 3-E shows that DMEM pre-incubation produced, at six hours p.i., a consistent increase in the proportion of cultured cells bearing FAS-positive lesions, quantified as an increase from 50 % to 100 % in the cases of strains EC218 and 140065. There may also have been an increase in the size of colonies and the intensity of the FAS, but inter-assay variation makes confident comparisons difficult. The AE capability of Strain EC222 $nal^r rif^r$ was only detected in the DMEM-induced assay. Comparisons between parent and antibiotic resistant derived strains in the DMEM-induced assay (Table 3-E) showed an attenuation of AE capability, to varying extents, for three of the four strain-pairs (140065/140065 nal^r, EC157/EC157 str^r, EC222/EC222 $nal^r rif^r$) tested.

Table 3-D: Observations on fluorescence actin stained inoculated HEp-2 monolayers

Strain	TVC*	Three hour incubation		Six hour incubation	
		Score†	Observations	Score†	Observations
B171	1.8	+	<10 % cells FAS+ colonies	+++	**Most cells colonised; large, bright FAS+ colonies**
85440	1.5	+/–	Occasional faint FAS+ colonies	++	FAS + colonies on about 50 % of cells
131567	1.6	–		+	Occasional FAS+ colonies
134107	0.98	–		+	Occasional FAS+ colonies
138345	1.3	–		+	Occasional FAS+ colonies
139229	1.4	–		+	Occasional FAS+ colonies
139579	1.3	+/–	Occasional small, faint FAS+ colonies	+	**FAS+ colonies on a minority of cells**
139969	1.0	–		+/–	Occasional, faint FAS
140019	1.2	+/–	**Occasional small, faint FAS+ colonies**	+	Dim FAS on approx. 20 % of cells
EC218	1.3	–	**Some fluorescent foci but no clear colonies**	++	**FAS+ colonies on approx. 50 % of cells**
EC218 *eaeA*⁻	1.2	–		–	
EC218 rif^r		X		++	
EC222		–		+	
EC222 nal^{r}rif^r		X		–	
140065	1.3	–		+	Dim FAS+ colonies on approx. 50 % of cells
140065 nal^r		X		+	
EC157		–		+	
EC157 str^r		X		+	

*TVC: total viable count of inoculum, x 10^7 cfu.
†Score: Subjective, semiquantitative, five-point score of FAS intensity and extent
'X' indicates data unavailable, as three-hour assays were not performed.
Bold entries are illustrated in Figure 3-h.
Strains in the lower part of the table are those used in subsequent animal experiments (chapters 4 –6)

Table 3-E: Observations on fluorescence actin stained HEp-2 monolayers incubated for six hours with *E. coli* O157:H7 strains pre-incubated in DMEM

Strain	Inoculum TVC (cfu)	FAS-positive features	
		Score*	Comments
EC218 WT	9.8×10^7	4	Approx. 1 large microcolony per cell
EC218 rif^r	7.7×10^7	4	Similar to WT
EC222 WT	1.1×10^8	2	Small to moderate microcolonies on approx. 50 % of cells
EC222 $nal^r rif^r$	6.2×10^7	1	Very small, rare microcolonies, <<1 per field
140065 WT	8.2×10^7	5	Large microcolonies on virtually all cells
140065 nal^r	1.3×10^8	4	Moderate to large microcolonies on virtually all cells
EC157 WT	1.2×10^8	4	Moderate to large microcolonies on virtually all cells
EC157 str^r	1.2×10^8	3	Slightly fewer and smaller microcolonies than WT

WT: Wild-type. * Semiquantitative 6-point comparative scale of extent of FAS lesions: 0 (no FAS lesions) - 5 (maximal extent of FAS lesions seen in this set).

Comparison of DMEM and PBS pre-incubation upon fluorescence actin staining of *E. coli* O157:H7 and O26:H11 strains on HEp-2 cells

To further investigate the apparent differences between *E. coli* O157:H7 and O26:H11 responses to DMEM induction seen in the intestinal loop experiments (Chapter 4), FAS was performed using PBS and DMEM incubation prior to inoculation of HEp-2 monolayers, as described in Section 2.5.5.4. Results are summarised in Table 3-F. A subjective 6-point semiquantitative score, combining size and frequency of FAS-positive lesions, as used previously (Table 3-E) was assigned to each stained monolayer. Using this score and a one hour pre-incubation in PBS as a baseline, a three-hour pre-incubation in DMEM had a positive or neutral effect upon the formation of FAS-positive lesions for all *E. coli* O157:H7 strains, whereas the EC1537 O26:H11 strain was not enhanced and indeed appeared to be inhibited by the DMEM treatment.

Table 3-F: Fluorescence actin staining of inoculated HEp-2 cells, comparing PBS and DMEM pre-incubation of *E. coli*

Preparation	Strain	Inoculum TVC*(cfu)	FAS	
			Score†	Comments
DMEM 3 hour incubation	EC218	9.8×10^7	4	Approx. 1 large microcolony per cell
	EC222	1.1×10^8	2	Small to moderate microcolonies on approx. 50 % of cells
	140065	8.2×10^7	5	Large microcolonies on virtually all cells
	EC157	1.2×10^8	4	Moderate to large microcolonies on virtually all cells
	NCTC 12900	7.3×10^7	5	Large microcolonies on all cells
	EC1537	6.7×10^7	2	Approx. 50 % of cells have small or moderate microcolonies
PBS 1 hour incubation	EC218	3.2×10^7	4	Similar to EC218 DMEM
	EC222	8.0×10^7	2	Small microcolonies on approx. 30 % of cells
	140065	1.2×10^8	4	Moderate to large microcolonies on virtually all cells
	EC157	1.75×10^8	3	Moderate colonies on approx. 90 % of cells
	NCTC 12900	9.7×10^7	5	Similar to NCTC 12900 DMEM
	EC1537	3.8×10^7	3	Small to large microcolonies on approx. 80 % of cells

†Semiquantitative 6-point comparative scale of extent of FAS lesions:
0 (no FAS lesions) - 5 (maximal extent of FAS lesions seen in this set).
*TVC: total viable count of the inoculum.
The data for the STEC O157:H7 strains in the upper part of the table is the same as in Table 3-E, as these assays were performed in parallel, but is presented also in this table for ease of comparison.

D. Scanning electron microscopy

SEM was performed on inoculated HEp-2 monolayers incubated for three and six hours (sections 2.5.5.5 and 2.7.4.1). The findings are summarised in Table 3-G and Figure 3-i. Strain B171 (EPEC) formed the most dense, rounded colonies, with microcolony formation clearly evident by three hours. The strains lacking *bfpA* did not form such compact, round microcolonies, and only one out of four *E. coli* O157:H7 strains (139579) showed appreciable microcolony formation by three hours. All strains showed identifiable microcolonies at six hours.

Table 3-G: Scanning EM observations of inoculated HEp-2 monolayers

Strain	Inoculum TVC*	Observations at three hours p.i.	Observations at six hours p.i.
B171	**1.8**	**Discrete, raised microcolonies on most cells, plus some flattened, more diffuse bacterial aggregations**	**Dense, rounded, discrete microcolonies on all cells.**
85440	1.2	Loose aggregates of generally fewer than 20 bacteria on most cells.	**A range of colonial forms: loose aggregates of approx. 50 bacteria, small (approx. 50 bacteria) to moderate (100-200 bacteria) –sized flattened or raised dense colonies.**
EC218	1.3	Scattered single bacteria over all cells, no evidence of microcolony formation.	Small, rounded microcolonies (50-100 bacteria) on approx. 50 % of cells, scattered bacteria on others.
EC222	1.3	Clusters of 2-5 bacteria on some cells.	**Small, flattened microcolonies (15-70 bacteria) on some cells, loose aggregates on others.**
EC157	1.0	Scattered bacteria over all cells, including loose clusters of up to 6 bacteria.	Small, rounded microcolonies (50-100 bacteria) on <50 % of cells, scattered bacteria on others.
139579	0.95	**Loose aggregates of ≤10 bacteria on some cells, scattered bacteria on others.**	**Flattened, small microcolonies (≤100 bacteria) on many cells, loose aggregates on others.**

*TVC: Total viable count, x 10^7 cfu.
Bold entries are illustrated in Figure 3-i.

In a follow-up study, prompted as described in Section 3.5.2C for the FAS study, DMEM-induced cultures of the four strains in the mixed inoculum plus their parent strains were inoculated onto HEp-2 monolayers, in order to compare the SEM appearance of the parent strains and antibiotic resistance marked derivatives. The results are summarised in Table 3-H. Like the comparative FAS studies (Section 3.5.2C), DMEM pre-incubation produced an apparent increase in the proportion of colonised cells by six hours p.i. in comparison with non-induced bacteria (Table 3-G), although the morphology and size of the colonies was not markedly different. When parent and antibiotic resistance marked strains were compared using DMEM pre-treatment, a reduction in colonisation was observed in three of four marked derivatives, shown by a reduction in colony size and/or the proportion of cells colonised. This effect was seen with the same three strain-pairs (EC222/ EC222 $nal^r rif^r$, 140065/140065 nal^r and EC157/EC157 str^r) that showed attenuation in the DMEM-induced FAS assay, and again Strain EC222 was the most markedly affected.

Table 3-H: Scanning EM observations on HEp-2 monolayers incubated for six hours with *E. coli* O157:H7 strains pre-incubated in DMEM for three hours

Strain	Inoculum TVC (cfu)	Observations at six hours p.i.
EC218 WT	9.8×10^7	Flattened microcolonies on most cells, some colonies are denser and rounded.
EC218 rif^r	7.7×10^7	Similar to WT, but no dense, rounded microcolonies seen.
EC222 WT	1.1×10^8	Most cells are colonised with flattened microcolonies.
EC222 $nal^r rif^r$	6.2×10^7	Scattered, single bacteria across the low-power field. One deeply-embedded at high power.
140065 WT	8.2×10^7	Extensive flattened microcolonies on all cells.
140065 nal^r	1.3×10^8	Microcolonies smaller than WT, but present on all cells, often dense and rounded up.
EC157 WT	1.2×10^8	Large flattened microcolonies on all cells, plus dense 'balls' with surface bacteria, resembling colonised cells which have become markedly contracted.
EC157 str^r	1.2×10^8	Microcolonies, smaller than for WT, present on most cells.

WT: wild-type

3.5.2.2 Bovine cell culture assays

The opportunity arose during the present studies to use bovine-derived epithelial-like intestinal cell cultures derived from a neonatal calf (Dibb-Fuller et al. 2001), which may be more similar phenotypically to ovine enterocytes than are the standard laboratory cell lines. Some of the cell association assays which had been done using HEp-2 cells were repeated using the bovine monolayers and the *E. coli* O157:H7 strains used in *in vivo* studies. Three cell cultures, derived from ileum, colon and rectum were chosen, reflecting possible colonisation sites in the ruminant host, as suggested by studies with neonatal and older calves (Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1999).

A. Quantitative association assay

Quantitative association assays were performed on semiconfluent monolayers of all three cell cultures (sections 2.5.5.1 and 2.5.5.2). The results are shown in Figure 3-j. A consistent feature is the tendency for counts to increase with a distal progression from ileal to rectal cells. There are however no discernible differences between bacteria, either between strains or between wild-type and antibiotic resistant derivatives. Furthermore, the association of a laboratory K12 strain (DH5α) lacking the LEE, was comparable in this three-hour assay to the AEEC O157:H7 strains, suggesting that *E. coli* O157:H7-specific mechanisms of

adherence including AE lesions and associated processes such as EspA filament adhesion, were of minor or no significance to the overall number of adherent bacteria at this stage.

B. Giemsa stained preparations

Monolayers of all three bovine cell types were inoculated, fixed and stained as described in sections 2.5.5.1 and 2.5.5.3. The three-hour incubation was selected, to assist comparison with the quantitative association assay. Observations made under the light microscope are summarised in Table 3-I.

There was considerable variation in the appearance of the cultured cells, probably signifying a degree of degeneration before or during the assay. The main dichotomy was between 'non-staining' cells, showing a lack of nuclear stain with or without cytoplasmic pallor and vacuolation but no evidence of widespread apoptosis or shrinkage, and 'staining' cells, showing strong nuclear staining and moderate cytoplasmic staining but often appearing vacuolated, rounded up or disintegrating. Cells were in wide zones of one state or the other, with relatively narrow transitional zones, i.e. 'staining' and 'non-staining' cells were not admixed. Patterns of adhesion were varied and included diffuse, localised (microcolonial) and a pattern of bacterial concentration along intercellular boundaries resembling the 'log-jam' pattern described for *E. coli* O157:H7 strains on human ileocaecal (HCT-8) cells (McKee and O'Brien 1995b). The latter pattern is interesting, as the cells on which it was seen in the present study were from a similar anatomical location to those in the original report. Although the viability of the cells was doubtful and no strong trend of adhesion patterns is discernible, only 5 of 30 *E. coli* O157:H7 strain/cell-type combinations did not yield evidence of either clustered or 'log-jam'-type adhesion. By contrast, no pattern or evidence of preferential association with cells compared with coverslip glass was seen with any of the control *E. coli* K12-infected monolayers.

Table 3-I: Observations on Giemsa-stained inoculated bovine cell cultures at three hours p.i.

Strain	Ileum	Colon	Rectum
EC218 WT	70 % cells *NS*. Bacteria more on *staining* cells, some in loose and dense 'log jam'-type patterns.	30 % cells *NS*. Bacteria common, some small clusters, more on *staining* cells.	80 % cells *NS*. Bacteria common, some clustered, and strongly cell-associated.
EC218 rif^r	70 % cells *NS*. Scattered bacteria, more on *staining* cells, sparse, small flat clusters.	40 % cells *NS*. Bacteria common, some small clusters. Similar numbers on all cells.	90 % cells *NS*. Bacteria common, often clustered, and strongly cell-associated.
EC222 WT	70 % cells *NS*. Bacteria mostly in sparse, flat clusters, on both types of cell.	30 % cells *NS*. Bacteria scattered. Occasional small clusters, most on *NS* cells.	80 % cells *NS*. Bacteria common, much denser over *staining* cells, no pattern.
EC222 $nal^r rif^r$	60 % cells *NS*. Bacteria scattered, preferentially on *staining* cells where there are sparse, small clusters.	40 % cells *NS*. Scattered bacteria, sparse small clusters. Few on *NS* cells.	80-90 % cells *NS*. Bacteria common on *staining* cells, clusters common. Few bacteria over *NS* cells.
140065 WT	20-30 % peripheral cells *non-staining*. Bacteria predominantly scattered over *staining* cells, loose 'log jam' pattern. Few clusters.	20 % *NS* peripheral cells. Others typically vacuolated. Bacteria locally abundant, no microcolonial pattern, fewer on *NS* cells.	70 % cells *NS*. Bacteria scattered, on both cell types closely cell-associated, clusters predominate, mostly on *staining* cells.
140065 nal^r	30 % peripheral cells *NS*. Bacteria scattered and small-large flat clusters, on *staining* cells.	As 140065 WT	70 % cells *NS*. Bacteria common, more over *staining* cells, but no strong pattern.
EC157 WT	30 % peripheral cells *NS*. Bacteria sparse, infrequently clustered, no adhesion difference between cell types.	10 % peripheral cells *NS*. Bacteria locally abundant, tend to loose 'log-jam' pattern. Few on *NS* cells.	50 % *NS*. Bacteria plentiful, no pattern, similar density over both cell types.
EC157 str^r	50 % cells *NS*. Bacteria sparse, clearly more frequent over *staining* cells. No pattern.	50 % cells *NS*. Bacteria common, locally abundant, some in loose 'log-jam' pattern. Few on *NS* cells.	80 % cells *NS* / weakly-staining. Bacteria scattered, more common over *staining* cells, where occasionally clustered.
NCTC 12900	50 % cells *NS*. Bacteria mainly loose 'log-jam' pattern on *staining* cells, smaller numbers in small, loose aggregates on *NS* cells.	50 % cells *NS*. Bacteria common, adhering to both cell types equally. Clusters common. Adherence closely cell-associated (more than other strains).	>90 % cells *NS*. Bacteria common over *NS*, strongly clustered and cell-associated. *staining* cells too few & shrunken to assess meaningfully.
EC1537	50 % cells *NS*. *staining* cells often vacuolated. Bacteria sparse, mainly on *staining* cells, occasional small microcolonies.	>80 % cells *NS* or weakly-staining. Others shrunken and disintegrating. Bacteria common, weak 'log-jam' pattern, mostly uninterpretable.	80 % cells *NS*. Bacteria scattered, some clusters on cells, but little difference in density between *staining*, *NS* and glass.
K12 DH5α	70 % cells *NS*. Bacteria scattered across all cells - no pattern.	>90 % *NS* or weakly-staining. Bacteria common, not cell-associated.	100 % *NS*, Patchy bacteria, unclustered, similar density on cells and glass.

NS: *non-staining*. See Section 3.5.2.2B for explanations of *staining* and *non-staining*. WT: wild-type

C. Fluorescence actin staining

Bovine cell monolayers were inoculated with *E. coli* O157:H7, O26:H11 and K12 strains, incubated for six hours, and FAS was performed according to the protocols in sections 2.5.5.1and 2.5.5.4. The results are summarised in Table 3-J and in Figure 3-k.

In general, less than 50 % of coverslip area had attached cells. This was often much less, i.e. below 10 %. As cells were verified to be semi-confluent at the start of the inoculation procedure, substantial detachment of cells must have occurred at some stage(s) of incubation, washing and staining. The degree of cell loss and degeneration did not appear to be correlated with the presence of an STEC inoculum, and the rectum-derived cells were consistently the most markedly degenerated. The lesions were subjectively scored, as previously described (Table 3-E), and all AE strains proved FAS-positive on all cell types. There appears to be no pattern of susceptibility to FAS-positive colonisation by *E. coli* O157:H7 amongst the three cell types, and the small intestine-derived (ileal) cells yielded similar scores to the large intestine-derived cells. Moreover, when considering bacterial strains, no positive correlation is evident between the degree of FAS-positive colonisation with this technique and the strain persistence characteristics in lambs (Chapter 6).

Table 3-J: Observations on fluorescence actin staining of inoculated bovine cell cultures *Continued on page 98*

Strain	TVC*	Cell Type	Score†	Comments
EC218	9.0	Ileum	4	FAS+ MC, typically 50-100 bacteria, on 100 % of cells. Cells generally well preserved.
		Colon	4	Small to large FAS+ MC on 100 % of viable cells. Approximately 50 % of cells are rounded up and degenerate.
		Rectum	4	FAS+ MC, typically medium or large (100 bacteria) on 90 % of cells. Approximately 70 % of cells are rounded up and degenerate.
EC218 rif^r	8.2	Ileum	3	FAS+ MC, typically 10-50 bacteria, some up to 100, on 70 % of cells. Cells generally well preserved.
		Colon	3	Small and medium FAS+ MC present on 80 % of viable cells. Approximately 30 % of cells are rounded up and degenerate.
		Rectum	**2**	**Small or medium FAS+ MC present on 50 % of viable cells. Approximately 80 % of cells are rounded up and degenerate.**
EC222	7.8	Ileum	1	FAS+ MC, comprising 5-20 bacteria, are rare, on <<10 % of cells. Approximately 50 % of cells are rounded up and degenerate.
		Colon	2	Small to medium FAS+ MC present on 30 % of viable cells. Approximately 70 % of cells are rounded up and degenerate.
		Rectum	<4	Fewer than 10 viable cells seen. Small FAS+ MC seen on 2 cells.
EC222 $nal^r rif^r$	6.3	Ileum	1	FAS+ MC, comprising 10-20 bacteria, are present on up to 10 % of cells. Approximately 50 % of cells are rounded up and degenerate.
		Colon	1	Rare FAS+ clusters of typically 5 bacteria present. Approximately 50 % of cells are rounded up and degenerate.
		Rectum	**1**	**Small FAS+ MC present uncommonly on viable cells. Approximately 70 % of cells are rounded up and degenerate.**
EC157	7.0	Ileum	1	FAS+ MC, comprising up to 30 bacteria, are present on approximately 10 % of cells. Approximately 50 % of cells are rounded up and degenerate.
		Colon	3	Small FAS+ MC present on 90 % of viable cells. Approximately 50 % of cells are rounded up and degenerate.
		Rectum	2	Small FAS+ MC present on 50 % of viable cells. Approximately 80 % of cells are rounded up and degenerate.
EC157 str^r	7.8	Ileum	0	No FAS+ bacterial adherence sites seen. Approximately 50 % of cells are rounded up and degenerate.
		Colon	1	Small FAS+ MC are present but very rare. Approximately 50 % of cells are rounded up and degenerate.
		Rectum	(0)‡	No FAS+ bacterial adherence sites seen. Poor quality samples, as approximately 90 % of cells are rounded up and degenerate.

Notes: see page 98

Table 3-J continued

Strain	TVC*	Cell Type	Score†	Comments
140065	8.3	Ileum	4	FAS+ MC of up to 200 bacteria present on 100 % of viable cells. Approximately 50 % of cells are rounded up and degenerate.
		Colon	4	Small to large FAS+ MC are present, on 100 % of viable cells. Approximately 50 % of cells are rounded up and degenerate.
		Rectum	3	Small and medium FAS+ MC, sometimes very loose, present on 100 % of viable cells. 60-70 % of cells are rounded up and degenerate.
140065 nal^r	4.8	Ileum	1	FAS+ MC, typically of 10 bacteria, present on up to 10 % of viable cells. Approximately 50 % of cells are rounded up and degenerate.
		Colon	2	Small FAS+ MC are rare overall, but present on up to 30 % of cells locally. Approximately 50 % of cells are rounded up and degenerate.
		Rectum	1	Scattered, small FAS+ MC on viable cells. Approximately 80 % of cells are rounded up and degenerate.
NCTC 12900	8.2	Ileum	4	FAS+ MC of up to 200 bacteria, on 90 % of cells. The majority of cells appear viable.
		Colon	**5**	**Large FAS+ MC are present on 100 % of viable cells. Cells typically have multiple MC. Approximately 50 % of cells are rounded up and degenerate.**
		Rectum	4	Medium and large FAS+ MC present on 100 % of viable cells. Slightly less florid than same strain on colon cells. Approximately 60 % of cells are rounded up and degenerate.
EC1537	4.8	**Ileum**	**2**	**FAS+ clusters, looser than the O157 MC and comprising 10-30 bacteria, on 50 % of cells. The majority of cells appear viable.**
		Colon	2	Small to medium FAS+ MC, looser than O157 MC, are present on 50 % of viable cells. Approximately 70 % of cells are rounded up and degenerate.
		Rectum	3	Small and medium FAS+ MC present on 70 % of viable cells. Approximately 90 % of cells are rounded up and degenerate.
K12 (DH5α)	4.8	Ileum	0	No FAS+ bacterial adherence sites seen. The majority of cells appear viable.
		Colon	0	The majority of cells are rounded up and degenerate.
		Rectum	0	Very few cells present, typically contracted and degenerate. No FAS+ bacterial adherence sites seen.
None	-	Ileum	0	No FAS+ bacterial adherence sites seen. Approximately 50 % of cells are rounded up and degenerate.
		Colon	0	Few cells present, most are rounded up and degenerate.
		Rectum	-	Very few cells present, almost all contracted and degenerate, or showing dissolution of the FITC-stained cytoskeletal actin. No FAS+ bacterial adherence sites seen.

*TVC: total viable count of the inoculated broth, x 10^8 cfu/ml.
†Semiquantitative 6-point comparative scale of extent of FAS lesions:
0 (no FAS lesions) - 5 (maximal extent of FAS lesions seen in this series).
‡ Score uncertain, as few cells present for evaluation.
MC: microcolonies; implies loose but discrete aggregations of bacteria.
Bold entries are illustrated in Figure 3-k.

3.5.3 Acid tolerance studies

3.5.3.1 Inorganic acid tolerance

The stationary phase acid resistance of candidate *E. coli* O157:H7 strains was assessed using hydrochloric acid at low pH (2.5) for 2.5 hours. This procedure was intended to simulate the environmental stress associated with passage through the monogastric stomach or the ruminant abomasum, and has been shown to discriminate between acid-sensitive and acid-resistant strains of enteric *E. coli* (Gorden and Small 1993), and stationary phase *E. coli* O157:H7 in particular (Waterman and Small 1996). A 10 % survival level, following previously published methods (Gorden and Small 1993; Waterman and Small 1996), was chosen to separate resistant and sensitive phenotypes.

The results of a single assay for each strain are presented in Table 3-K. The strains examined were thus markedly heterogeneous in respect of stationary phase inorganic acid tolerance.

Table 3-K: Inorganic acid tolerance of *E. coli* O157:H7 strains

Strain	% survival	Phenotype
EC218	0.0009	Sensitive
EC222	55	Resistant
EC157	0.04	Sensitive
131567	1	Sensitive
134107	4	Sensitive
138345	5	Sensitive
139229	42	Resistant
139579	0	Sensitive
139969	7	Sensitive
140019	18	Resistant
140065	70	Resistant

3.5.3.2 Acidic pH tolerance in the presence of volatile fatty acids

The inhibitory effect of VFA upon *E. coli*, including *E. coli* O157:H7, under acid conditions is well established (Wolin 1969; Rasmussen et al. 1993; Lin et al. 1996). Proposed mechanisms for this effect include the importation of protons (H^+) into the bacterial cell, with subsequent dissociation of the VFA-proton molecule and release of H^+ into the cytosol, and membrane disruption by the VFA molecule. These mechanisms would

lead to loss of internal pH homeostasis (Benjamin and Datta 1995; Booth et al. 1999) and the toxic intracellular accumulation of VFA anions (Russell 1992).

Various sources, reporting analyses of ovine intestinal contents under defined feeding regimes (Dougherty et al. 1975; Anon 1979; Church 1979; Maloiy and Clemens 1980; Lewis and Dehority 1985) provide information on the range of normal pH and VFA concentrations in the rumen and hindgut of sheep. The ranges of values derived from these are as follows:

<u>Large intestine</u>

pH:	minimum 6.0		(caecum, 60% grain diet; Lewis and Dehority 1985)
	maximum 8.1		(rectum, hay diet; Maloiy and Clemens 1980)
Total VFA: (mmol/l)	minimum 44		(rectum, grass diet; Lewis and Dehority 1985)
	maximum 160		(caecum, 60% grain diet; Lewis and Dehority 1985)
VFA composition: (molar percentages)	Acetate	71 - 85	(Lewis and Dehority 1985)
	Propionate	9.6 - 14.5	
	Butyrate	2.3 - 17.5	
	Valerate	0.8 - 4.2	

Rumen

pH:	5.5 – 7.0		(Anon 1979)
Total VFA: (mmol/l)	60 – 120		(Anon 1979)
VFA composition: (molar percentages)	Acetate	approx. 60	(Church 1979)
	Propionate	approx. 20	
	Butyrate	approx. 15	
	Valerate	approx. 1-6	

With reference to the above reports, it was decided to use a standard mixture of acetate, propionate, butyrate and valerate in a molar ratio of 77:10:10:3, at three different overall concentrations (0, 100 and 150 mmol/l) in order to span the range likely to be encountered within the ovine alimentary tract. Similarly, the three pH values of 6.0, 6.5 and 7.0 were selected, to reflect the pH values quoted in the sources and to provide the mildly acidic conditions at which VFA-specific inhibition may be evident. Thus, for each strain tested, nine different combinations of VFA concentration plus pH were assessed, including the effects of pH alone (no added VFA) and of VFA at neutral pH. A standard, non-limiting nutrient source (LB-G broth) was chosen as the medium in which to create the VFA and pH

conditions and, as far as was practicable using gas jars, anaerobic culture conditions were employed, to reflect the environment in the alimentary tract. An initial bacterial concentration of approximately 10^5 cfu/ml was selected, in order to provide adequate scope for detectable growth or a reduction in viable bacteria over the eight hours of the assay. The assays were performed as described in Section 2.5.2.2, and the results for a single assay for each strain and VFA plus pH combination are summarised in figures 3-l, 3-m and 3-n.

In the absence of added VFA, all strains and their antibiotic resistant derivatives grew similarly within the pH 6 to 7 range, and the graphs are consistent with a sigmoid growth curve. With added VFA at 100 mmol/l, suppression of growth of all strains was evident at all pH values, but it was most marked at pH 6, where a reduction in growth of approximately two $\log_{10}$ units was seen by eight hours. Furthermore, the double-marked EC222 nal^{r}rif^r strain proved most susceptible to VFA-induced growth suppression, with growth at eight hours being approximately one $\log_{10}$ unit lower at all pH values than that of its parent strain EC222. At the highest VFA concentration of 150 mmol/l the effect was more pronounced. A further reduction in growth at eight hours of one to two $\log_{10}$ units at pH 6 amounted to a cessation of detectable growth in some cases. Growth at a reduced rate continued at pH 6.5 and 7. Additionally, the values at five hours suggested a prolonged lag phase in comparison with the previous graphs.

3.5.4 Growth in single and mixed broth culture

Following initial selection of strains for *in vivo* use (Section 3.6), the growth curves of each antibiotic resistance marked strain in LB-G broth were determined, initially in single-strain culture with photometric measurement of cell density, and subsequently in mixed culture with periodic TVC monitoring of cell density using antibiotic-supplemented nutrient agar (Section 2.5.3). The results (Figure 3-o, i. and ii.) show that, although all strains grew similarly in separate cultures, in mixed culture the detected growth of Strain 139579 spec^r ceased whilst the other strains were in mid-logarithmic phase, resulting in the final stationary-phase TVC for Strain 139579 spec^r being more than one $\log_{10}$ unit lower than the TVC of any other strain in the mixture. Strain 139579 spec^r was therefore substituted with Strain 140065 nal^r, and Strain EC222 nal^r was double-marked (EC222 nal^{r}rif^r) to permit differentiation, as further discussed in sections 3.4 and 3.6. These four strains were then grown separately and together in broth culture (Figure 3-o, iii. and iv. respectively.) and were also cross-streaked on nutrient agar plates (Section 2.5.3). The double-marked EC222 nal^{r}rif^r strain proved to be slower-growing in both culture experiments and achieved a final concentration between half and one $\log_{10}$ unit lower than the other three strains in mixed culture. However, the marked growth arrest seen in mixed culture with Strain

139579 spec^r was not seen with any strain. No growth inhibition was seen between any strains in the cross-streaking experiment.

3.6 Selection of strains for *in vivo* studies

In the absence of a comprehensive understanding of *E. coli* O157:H7 interactions with the carrier host, some unknown or unsuspected bacterial factor may be of significant importance in the process of attachment. As such a factor may vary between strains, it was considered that a mixed inoculum for *in vivo* studies would increase the likelihood that a strain or strains capable of attaching in some way would be inoculated, and form attachments. Bovine and human-derived strains were selected in order to include, respectively, a source associated with persistent ruminant strains and a source of strains which were virulent in humans and which may successfully have passed through the human food chain from an animal reservoir. Persistent sheep-derived *E. coli* O157:H7 were not readily available at the time for consideration as candidate strains.

The possible contribution of established virulence factors of *E. coli* O157:H7 to persistence in the field is unknown, so candidate strains were screened for established virulence-associated genes and for associated phenotypic characteristics. Using the results of genotypic and some phenotypic characterisations, four *E. coli* O157:H7 strains (EC218, EC222, EC157 and 139579) were selected initially for *in vivo* studies. Criteria for selection included: the presence of *eaeA* and four putative virulence factors located on the pO157 virulence plasmid and encoded by the *etpD*, *katP*, and *espP* genes and the *EHEC-hly* gene cluster (Section 3.3); *in vitro* intimin expression following DMEM induction (Section 3.5.1A); *in vitro* cell association comparable with other *E. coli* O157:H7 strains (Section 3.5.2.1A); and AE (FAS) capability in the six hour HEp-2 assay (Section 3.5.2.1C). Following PCR amplification of the entire *rpoS* stationary phase sigma factor gene (Table 2-A, Figure 3-a), none of the candidate strains were seen to have a gross lesion in this gene. The *rpoS* gene has been shown to be highly mutable in *E. coli*, including O157:H7, with small (Waterman and Small 1996) and large (Cookson, A., personal communication) genetic lesions having been found to be associated with a reduction in acid tolerance.

Variable features of the mixed inoculum were: source of the strain, inorganic acid tolerance and Shiga toxin subtype, these being characteristics which are observed to vary substantially in field strains of *E. coli* O157:H7 (Griffin and Tauxe 1991; Waterman and Small 1996). Following initial selection, the four strains were further characterised in respect of cell association by Giemsa-stained preparations and SEM examination (sections 3.5.2.1B and 3.5.2.1D respectively). Thus, the strains selected had a demonstrable capacity to adhere

to HEp-2 cells in a localised pattern and formed AE lesions, doing both in a manner typical of the EHEC O157:H7 pathotype (Nataro and Kaper 1998).

When the antibiotic resistance marked strains were grown in mixed broth culture (Section 3.5.4), detectable growth of Strain 139579 spec^r apparently ceased when the other strains were in mid-logarithmic phase, resulting in the final TVC for Strain 139579 spec^r being more than one log_{10} unit lower than the TVC of any other strain in the mixture. Therefore, at this stage it was replaced by the bovine-derived Strain 140065 nal^r, and Strain EC222 nal^r was additionally labelled with rif resistance, to distinguish between the two. This was done in order to be able to produce a four-strain antibiotic resistance marked mixture in which each strain would grow to an approximately equal density. Double-marking one strain was performed because a single-marked 140065 spec^r strain had an atypical small colonial appearance (Section 3.4). Cross-streaking and mixed batch culture showed no evidence of inter-strain growth inhibition with the combination of EC218 rif^r, EC222 nal^{r}rif^r, 140065 nal^r and EC157 str^r.

Following the substitution of Strain 139579 by 140065, Giemsa-stained (Table 3-C) and SEM (Table 3-H) preparations of the latter strain were examined. Strain 140065 had similar characteristics to the other three mixed inoculum strains in these assays. The shared and variable features of the strains in the mixed inoculum are summarised in tables 3-L and 3-M.

Table 3-L: Shared features of the component strains of the four-strain mixture

Strain	pO157 genes[1]	rpoS[2]	Intimin expression[3]	FAS[4]		HEp-2 pattern	
				3 hrs	6 hrs	Giemsa[5]	SEM[6]
EC218	*hly, espP, katP, etpD*	All with normal product size	All detected	–	++	Localised adherence	Small micro-colonies
EC222				–	+		
EC157				–	+		
140065				–	+		

1 & 2: Section 3.3; 3: Section 3.5.1A; 4: Section 3.5.2C; 5: Section 3.5.2B; 6: Section 3.5.2D.

Table 3-M: Variable features of the component strains of the four-strain mixture

Strain	Source	*stx* type[1]	Acid tolerance[2]
EC218	Bovine	1 & 2	Sensitive
EC222	Bovine	2	Resistant
EC157	Human	1 & 2	Sensitive
140065	Human	2	Resistant

1: Section 3.3; 2: Section 3.5.3.1

3.7 Discussion

The aims of the present *in vitro* studies were: the selection and characterisation of *E. coli* O157:H7 strains for *in vivo* studies, and the further characterisation of these and additional strains to complement findings from orally inoculated lambs and ligated ovine large intestinal loops.

Adhesion to HEp-2 cells, as measured by the three-hour quantitative association assay, was similar between all *E. coli* O157:H7 strains tested. Previous studies (Sherman et al. 1987; Donnenberg et al. 1989; McKee and O'Brien 1995b; Dibb-Fuller et al. 2001) have shown that *E. coli* O157:H7 does not invade HEp-2 cells to a significant degree, when compared with invasive bacteria such as EIEC and *Salmonella* species. Therefore, the quantitative association assays may be considered a reasonable reflection of the cell-adhesion qualities of the test *E. coli* O157:H7 strains. Some reports indicate that a three-hour incubation time is inadequate for the characterisation and quantification of EHEC adherence to cell cultures (Karch et al. 1987; Donnenberg and Nataro 1995). However, these reports concern assays using the microscopic examination of stained monolayers for adherent bacteria, not the cultural method for quantification used in the present studies. For the microscopic technique, the detection and counting of colonies is optimised by a three-hour initial adhesion incubation followed by washing (to preserve the integrity of the monolayer; Francis et al. 1991) and a three-hour colony-development stage. For quantifying adhesion by culture, the second three-hour incubation following washing merely results in multiplication of bacteria already adherent and is unlikely to aid the discriminating power of the assay.

Examination of certain strains including a human EPEC (B171), a bovine EPEC (85440) and various human- and bovine-derived *E. coli* O157:H7 strains on inoculated HEp-2 monolayers by light, fluorescence and electron microscopy yielded results which correlated closely between the different techniques. Strain B171 consistently formed dense microcolonies, which were FAS-positive (indicating AE lesion formation) by three hours p.i. Strain 85440 formed less dense, more flattened colonies at a slower rate and on a smaller

proportion of cells. The *E. coli* O157:H7 strains all exhibited the type of LA typical of the pathotype (McKee and O'Brien 1995b; Tatsuno et al. 2000) and characterised by the relatively slow formation (over more than three hours) of microcolonies which were of low density in comparison to those formed by typical (EAF-encoding) EPEC. Indeed, the *E. coli* O157:H7 LA resembles the 'poor LA' (Knutton et al. 1991) or 'LA-like' (Scaletsky et al. 1999) phenotype reported by other workers for atypical EPEC which lack EAF and, by inference, BFP also. The weak LA pattern seen with *E. coli* O157:H7 at three hours p.i. with the light microscope was generally not apparent at the higher magnification afforded by the scanning electron microscope. Adherence of the *E. coli* O157:H7 strains at three hours p.i. typically was not associated with detectable FAS.

The process of initial strain characterisation and selection has been discussed (Section 3.6). In the light of previous experience with an *E. coli* O157:H7 experimental strain (Cookson, A., personal communication), the *rpoS* stationary phase sigma factor genes of candidate strains were checked for lesions by PCR, whilst recognising that small changes undetectable by this method may still have rendered the gene non-functional in any particular strain. It was not originally intended to include in the mixed inoculum a strain (EC222 nal^{r}rif^r) for which no detectable FAS (AE) capability was evident in the standard assay. However, FAS studies using the marked *E. coli* O157:H7 derivatives were conducted in parallel with the *in vivo* studies, and it became apparent that Strain EC222 nal^{r}rif^r was substantially attenuated in respect of an AE capability in comparison with its parent strain only after first use of the four-strain inoculum. For consistency, the mixed inoculum continued to be used as originally constituted, and the range of AE capabilities amongst the four marked strains, which became evident following standard and DMEM pre-incubated FAS, was potentially of value to attempt correlations with strain persistence *in vivo*. Although double resistance marking may have been significant in the degree of attenuation of the AE capability of Strain EC222 nal^{r}rif^r, the findings of the protein expression studies (sections 3.5.1B and 3.5.1C) demonstrate that the wild-type Strain 222 secreted low quantities of proteins, including EspA, in comparison with the other strains. Thus it appears that the double antibiotic resistance marking was applied within an already weak AE background.

The attenuation of the colonising and AE capability *in vitro* of the antibiotic resistance marked derivatives of strains EC222, 140065 and EC157 varied according to the strain/resistance marker combination, and Strain 218 appeared to be little affected by marking for rif resistance. The protein expression studies comparing wild-type and derived antibiotic resistance marked strains showed a pattern of protein secretion and intimin expression which correlated with that observed in respect of *in vitro* colonising and AE capability. Detailed comparison shows that, of the protein profiles examined, intimin

expression most closely paralleled the phenotypic findings, i.e. Strain 218 (least attenuation under FAS and SEM examination associated with antibiotic resistance marking) showed least apparent reduction in intimin expression when resistance marked, and Strain 222 (most attenuation under FAS and SEM examination associated with antibiotic resistance marking) showed the most marked apparent reduction in intimin expression when resistance marked.

The reduction in protein secretion seen in the antibiotic resistance marked strains appeared to affect most of the protein bands present and not just the identifiable proteins encoded by the LEE, suggesting that the observed attenuation was not due to mechanisms specifically targeting regulation of the LEE. Likely mechanisms of spontaneous single-step resistance in the marked strains used are: mutations in the *rpsL* ribosomal protein S12 gene for streptomycin (Bryan 1984), mutations in the *rpoB* DNA-dependent RNA polymerase gene for rifampicin (Reynolds 2000), and mutations in *gyr* DNA gyrase genes for nalidixic acid (Piddock 1999). There is commonly a 'fitness cost' associated with the acquisition of antibiotic resistance by a bacterium, at least initially (Schrag et al. 1997; Reynolds 2000), which may be associated with a reduction in the efficiency of DNA replication (*gyr*), of transcription (*gyr*, *rpoB*) or of translation (*rpsL*). As the fitness cost incurred may vary between different mutants (Reynolds 2000) it is perhaps unsurprising that the reduction in protein expression and the phenotypic attenuation varied substantially between the experimental strains.

When strains were grown in DMEM before the various cell association assays, colonisation and the formation of AE lesions, as assessed by SEM and FAS, were increased. This parallels the previously reported effect of DMEM in the promotion of LEE protein expression (Ogierman et al. 2000). Indeed, in the case of Strain EC222 nal^{r}rif^r, pre-incubation in DMEM restored an AE capability to a detectable level in the HEp-2 FAS procedure.

One-hour PBS and five-hour DMEM incubation was performed on five *E. coli* O157:H7 strains and one *E. coli* O26:H11 strain, and an increase in the concentration of the LEE proteins EspA and intimin was observed with the latter treatment relative to the former, although the effect on intimin with *E. coli* O26:H11 was unclear, due to substantial variation in the bacterial cell concentrations between assays. For *E. coli* O157:H7 strains this effect was seen also when overall protein secretion was assessed. However the O26:H11 strain appeared to behave differently, as the level of many supernatant proteins with PBS incubation was equal to or higher than that with DMEM incubation. Nonetheless, this effect did not include the proteins tentatively identified as EspB and EspD on the Coomassie-stained gel, which were increased in the DMEM preparation. The difference in the secreted protein profiles between *E. coli* O157:H7 and O26:H11 strains without DMEM induction parallels the finding that FAS lesion formation on HEp-2 cells was unaffected or even

enhanced by PBS pre-incubation for the *E. coli* O26:H11 strain, in contrast to the *E. coli* O157:H7 strains which were generally enhanced by DMEM pre-incubation (Table 3-F).

These studies comparing PBS- and DMEM-incubated bacteria were prompted by findings *in vivo* (Chapter 4), which also suggested a possible difference between *E. coli* O157:H7 and O26:H11 responses to DMEM incubation. The resulting FAS data reinforces a hypothesis, generated after reviewing the results of the intestinal loops studies, that the AE phenotype of *E. coli* O157:H7 is enhanced to a greater degree than that of *E. coli* O26:H11 (Strain EC1537) by DMEM pre-treatment. However, the protein expression studies do not support a specific role for Esp secretion in this proposed effect, despite showing a difference between the serotypes in terms of overall supernatant protein levels without DMEM induction. These were, however, limited studies and inter-strain comparisons are restricted by, for example, consideration that the growth phases of the strains were not monitored, and that protein expression at different stages of growth were not considered. Furthermore, such effects as cell contact-dependent secretion of Esp proteins (Beltrametti et al. 1999) were not examined. The relative contributions of media and temperature are also unclear in these studies, as temperature has been shown to affect markedly the secretion of EspB, and probably EspA, by *E. coli* O26:H- (Ebel et al. 1996) and EPEC O127:H6 (Kenny et al. 1997a).

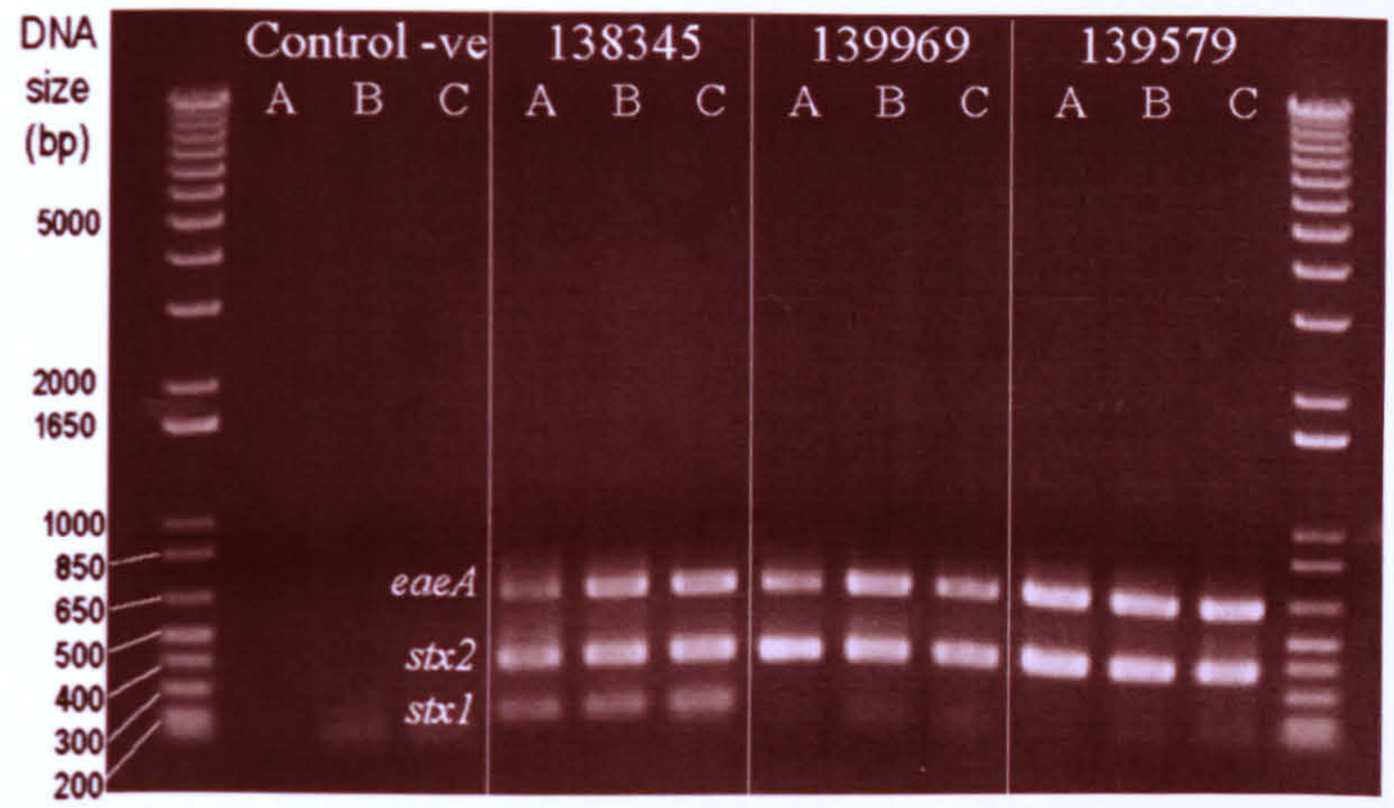
The use of standard laboratory epithelial-like cell lines, of both intestinal (e.g. Caco-2) and non-intestinal (e.g. HEp-2, HeLa) derivation is well established, and provides a degree of standardisation with which to compare the findings of different workers. However, the surface properties and physiological apparatus of cells from different species and body locations differ. Where an *in vitro* approach is being used to investigate an *in vivo* interaction between micro-organisms and host, it may be helpful to use cells which are as closely related to the host as possible. Using bovine intestine-derived cell cultures, no differences were observed between *E. coli* O157:H7 , O26 and K12 strains in an association assay. The use of a mannose-free incomplete medium for this assay may have complicated interpretation, as the control K12 strain may have expressed type-1 fimbriae whereas the *E. coli* O157:H7 strains would be unlikely to have done so (Section 1.4.2.2D). A difference between the cell types in the level of bacterial association was seen. However, as the cell cultures have proved more fragile in Giemsa-stained and FAS procedures than established cell lines such as HEp-2, this difference may have been due to differences in cell viability rather than in the adhesion characteristics of the cells *per se*.

When Giemsa- and FAS-stained bovine cell monolayers were examined three and six hours respectively after inoculation with *E. coli* O157:H7 strains, neither showed a correlation between adherence or AE lesions and the *in vivo* persistence characteristics of the

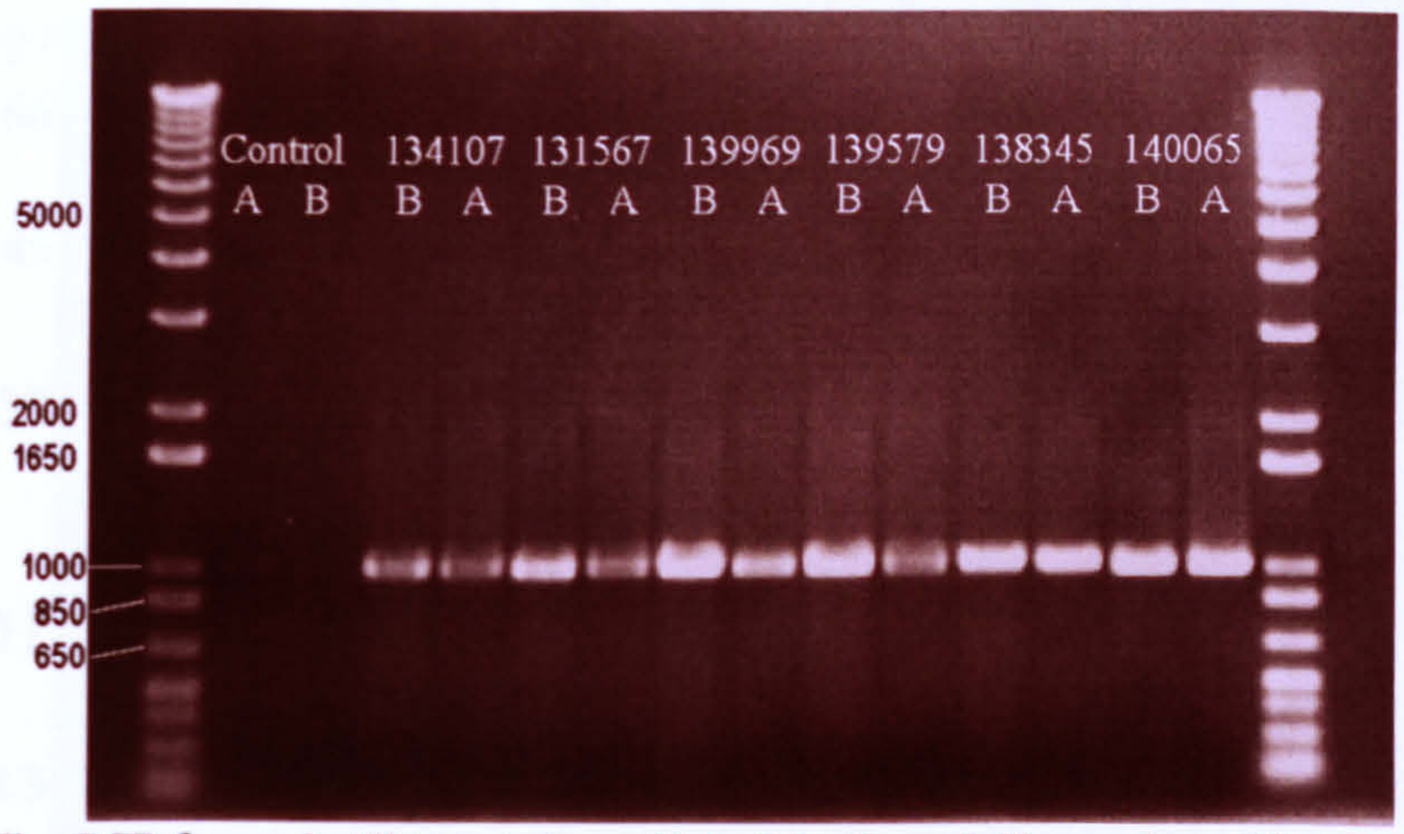
inoculum strain. Furthermore, a natural *E. coli* O26:H11 pathogen proved no more adherent to these bovine cells than did the *E. coli* O157:H7 strains.

The results comparing strain tolerances to VFA at acidic pH show the expected pattern, based upon previous work (Rasmussen et al. 1993), and do not demonstrate that one strain is clearly more tolerant of VFA at physiological alimentary pH values than another. The pronounced susceptibility of the double-marked Strain EC222 nal^{r}rif^r in the 100 mmol/l VFA assay is further evidence that such marking procedures places bacteria at a competitive disadvantage in some environments.

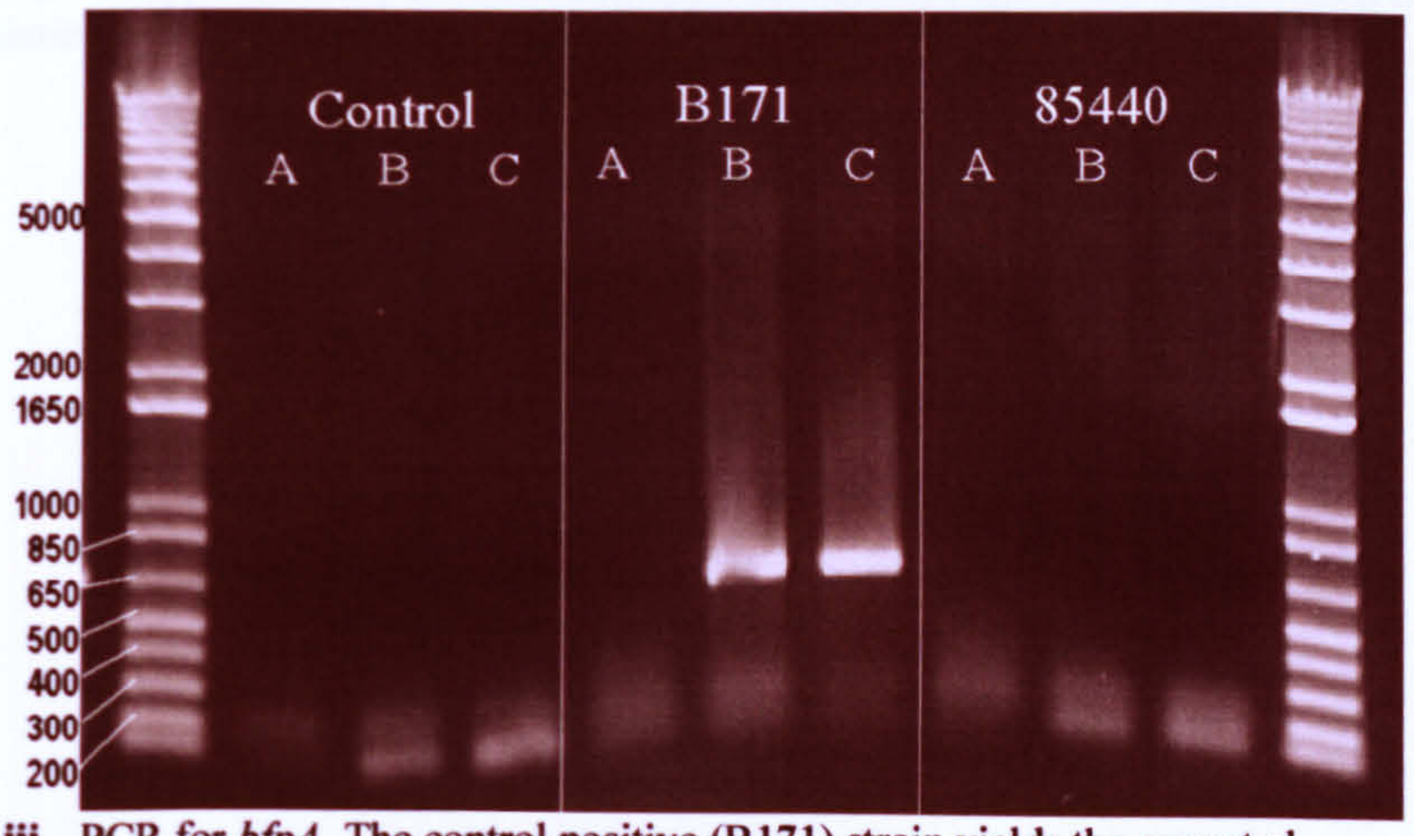
Figure 3-a: PCR reaction products for gene detection



i. Multiplex PCR. Bands of the expected size are present, indicating that all strains possess *eaeA*, two possess *stx2* and one possesses *stx1* and *stx2*. 'A', 'B' and 'C' indicate different PCR buffers.

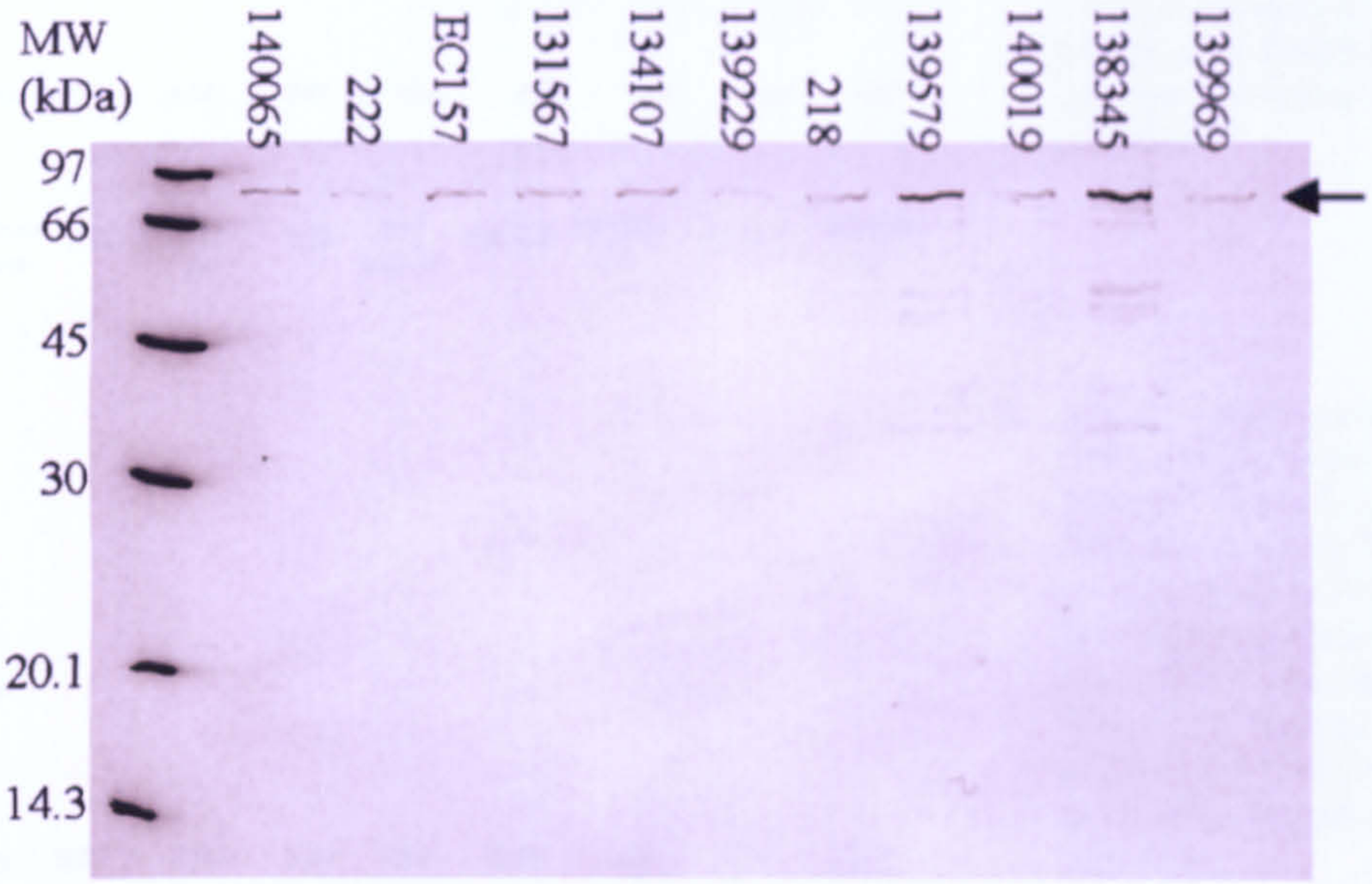


ii. PCR for *rpoS*. All test strains, with both buffers, yield a product of the same (expected) size. 'A' and 'B' indicate different PCR buffers.



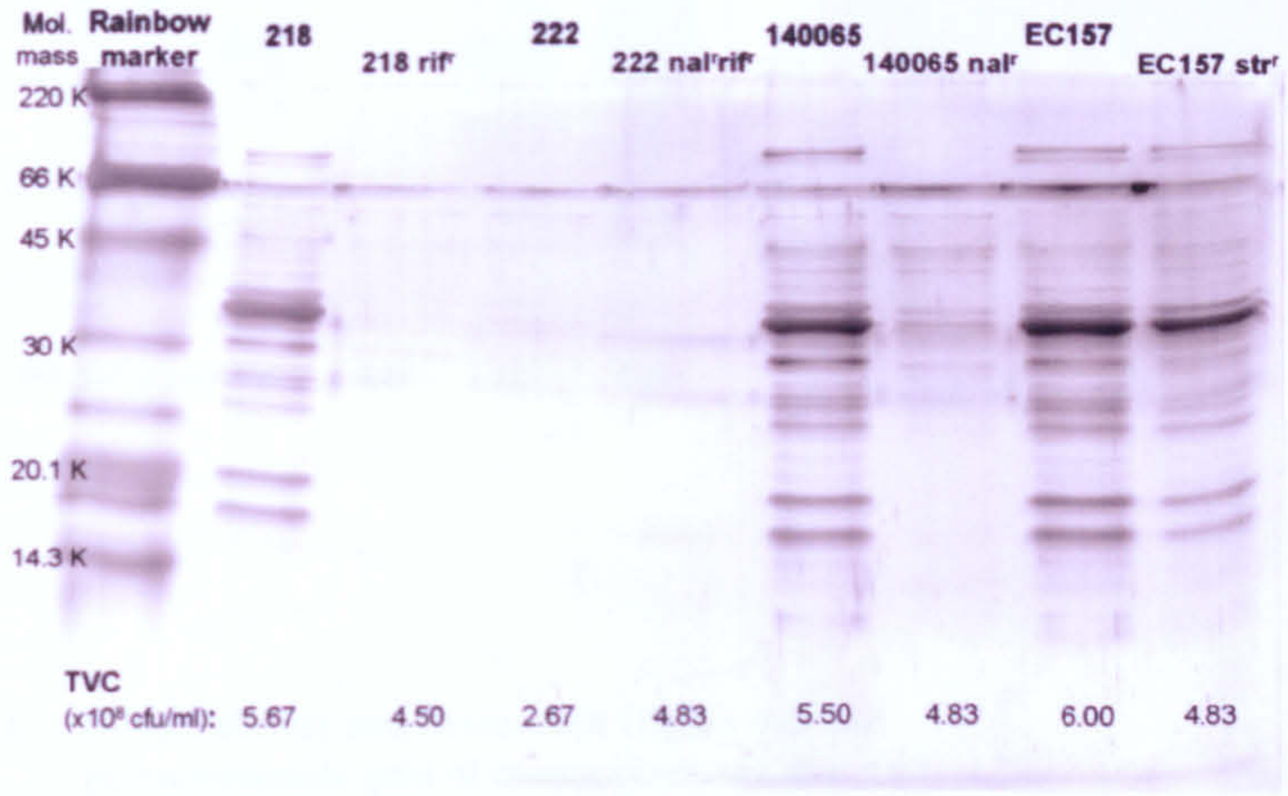
iii. PCR for *bfpA*. The control positive (B171) strain yields the expected product with two out of three PCR buffers, whilst the test (85440) strain yields no product. 'A', 'B' and 'C' indicate different PCR buffers.

Figure 3-b: Western blot of intimin from *E. coli* O157:H7 strains

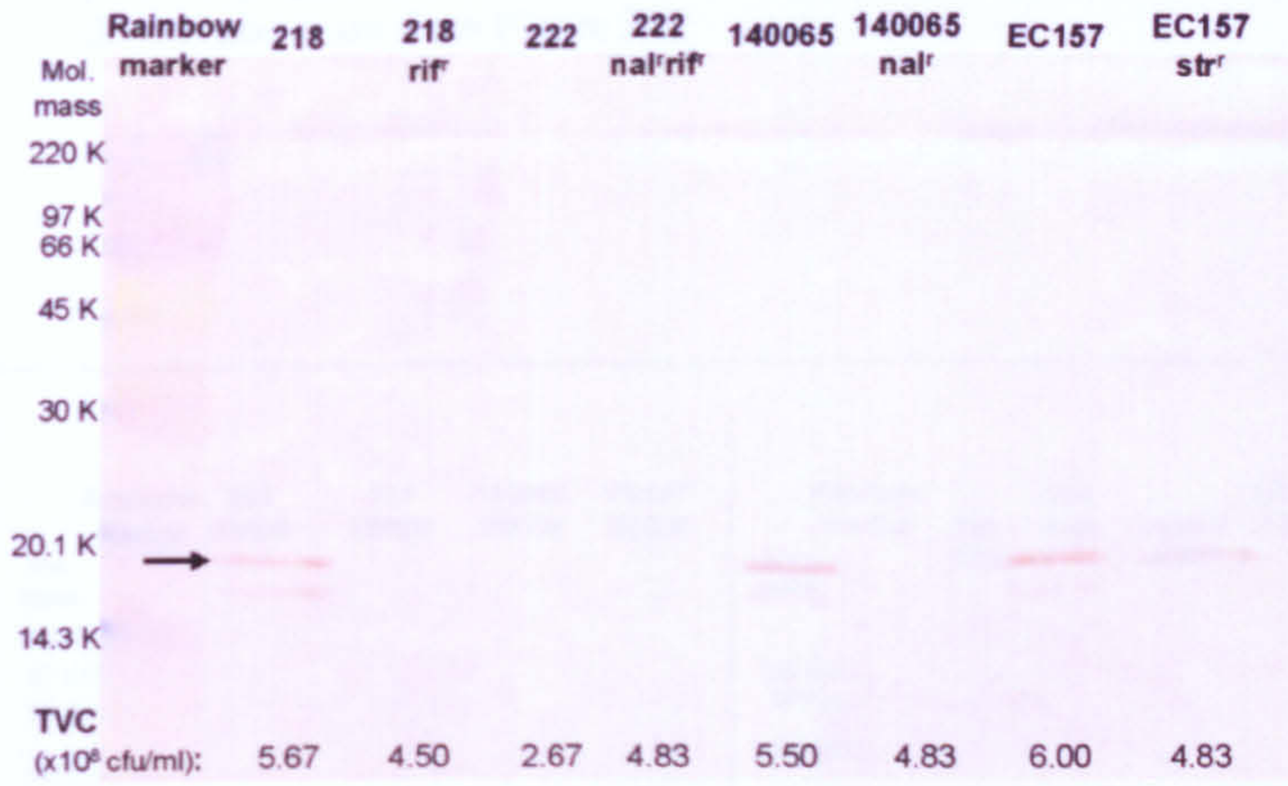


A band of approximately 94 kDa (arrowed), within the expected molecular weight range for intimin$_{O157}$, is detected for all strains tested.

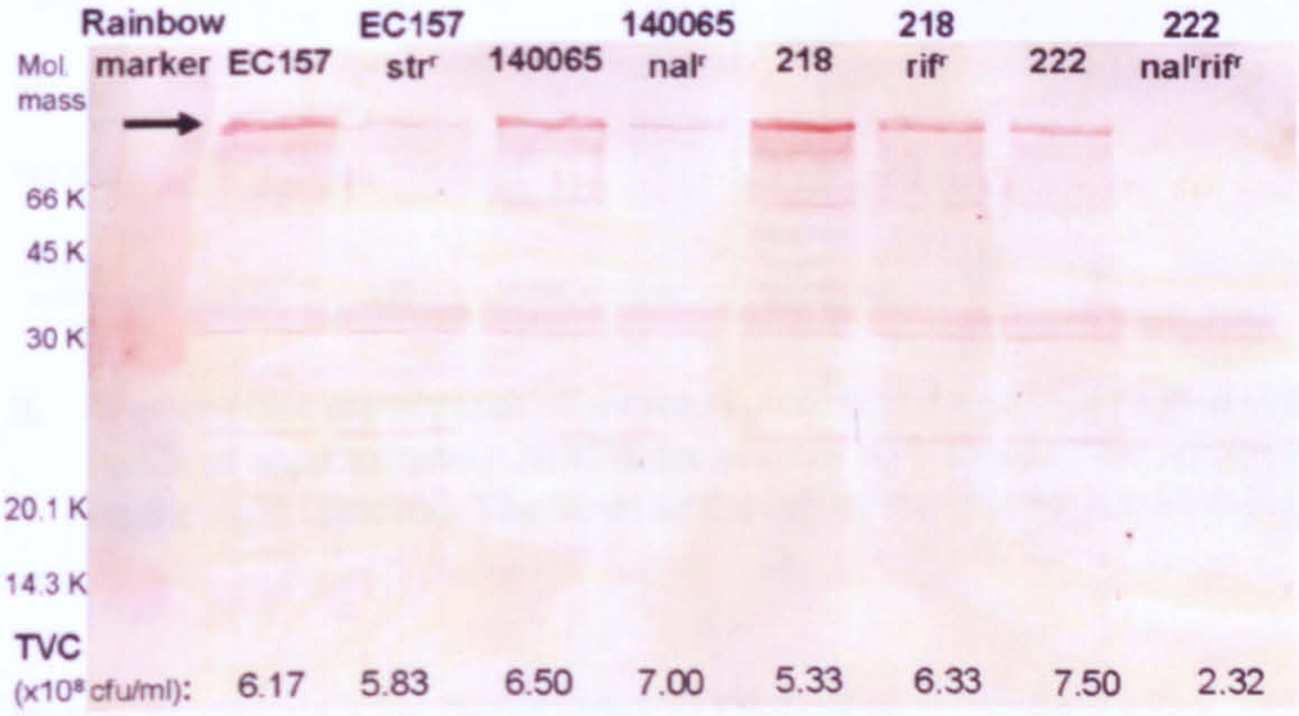
Figure 3-c: LEE protein expression from *E. coli* O157:H7 strains, following DMEM induction



i. Silver stained polyacrylamide gel of electrophoretically separated secreted protein preparations. The final TVC of the bacteria incubated in DMEM is given at the bottom of each lane.

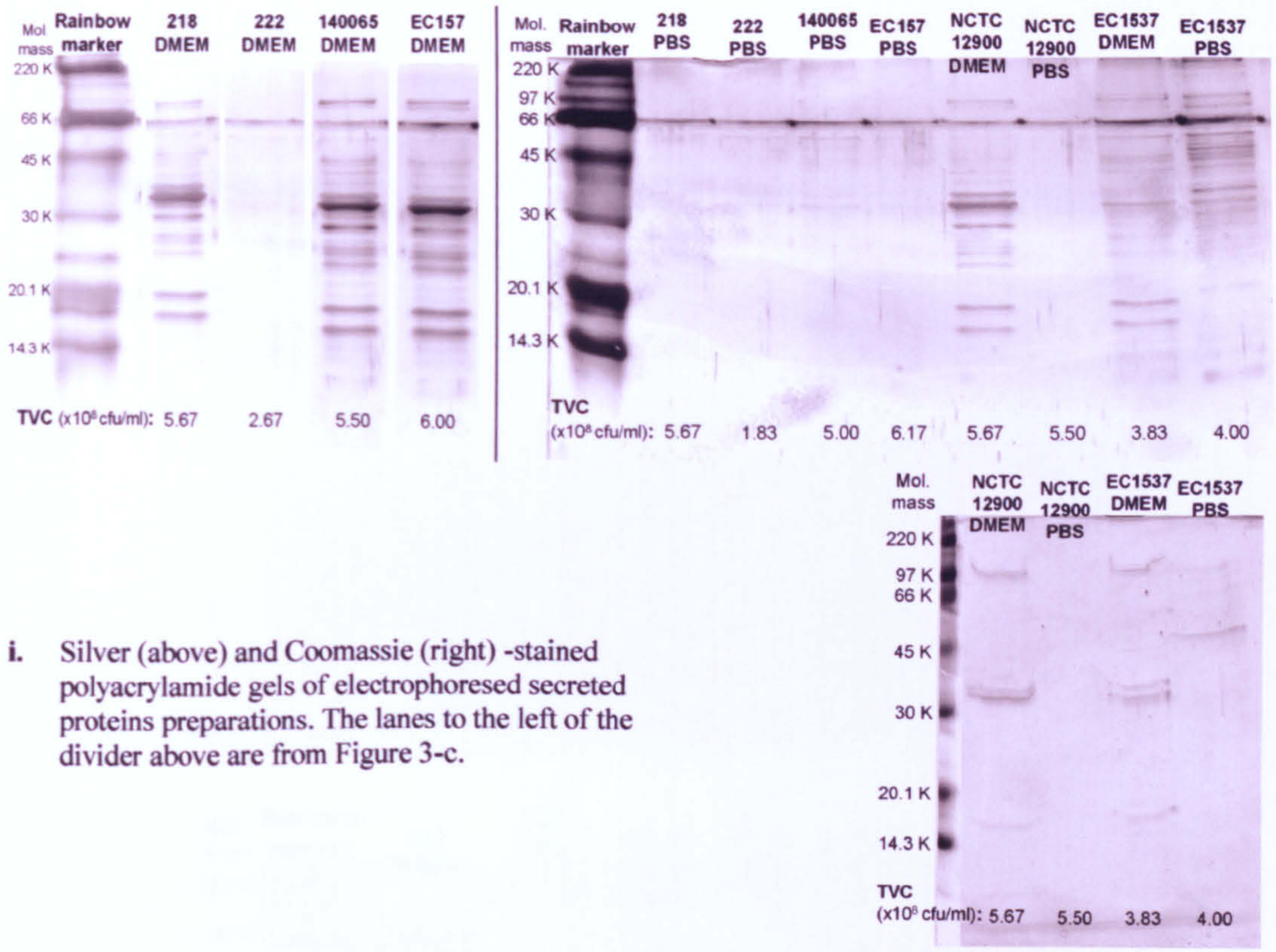


ii. Western blot of secreted protein preparations probed with $EspA_{O157}$ antibody. A double band of approximately 20 kDa (arrow) is present for some strains. The final TVC of the bacteria incubated in DMEM is given at the bottom of each lane.

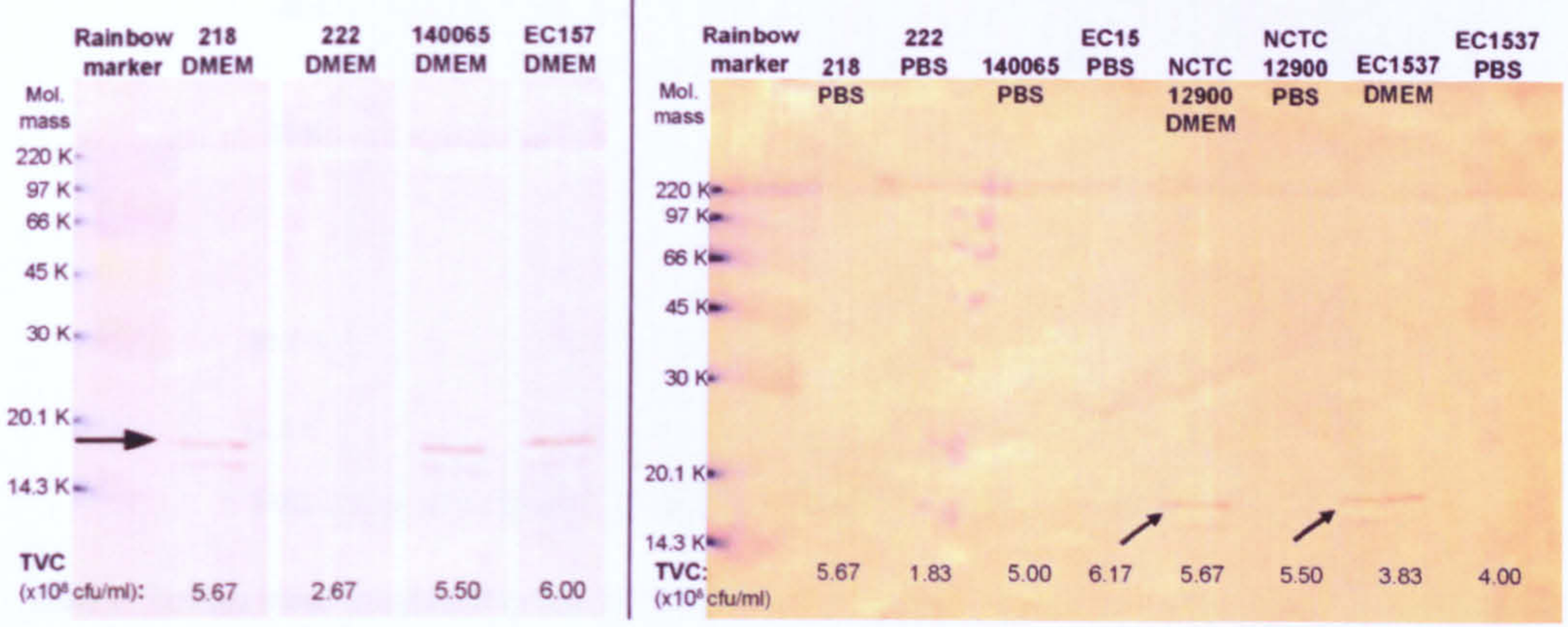


iii. Western blot of a cell lysate, previously incubated in DMEM, probed with an $intimin_{O157}$ antibody. An intense band at the expected 94-97 kDa mass for $intimin_{O157}$ (arrow) is present with most strains. The final TVC of the bacteria incubated in DMEM is given at the bottom of each lane.

Figure 3-d: Secreted protein profiles of *E. coli* O157 and O26 strains incubated in DMEM or PBS



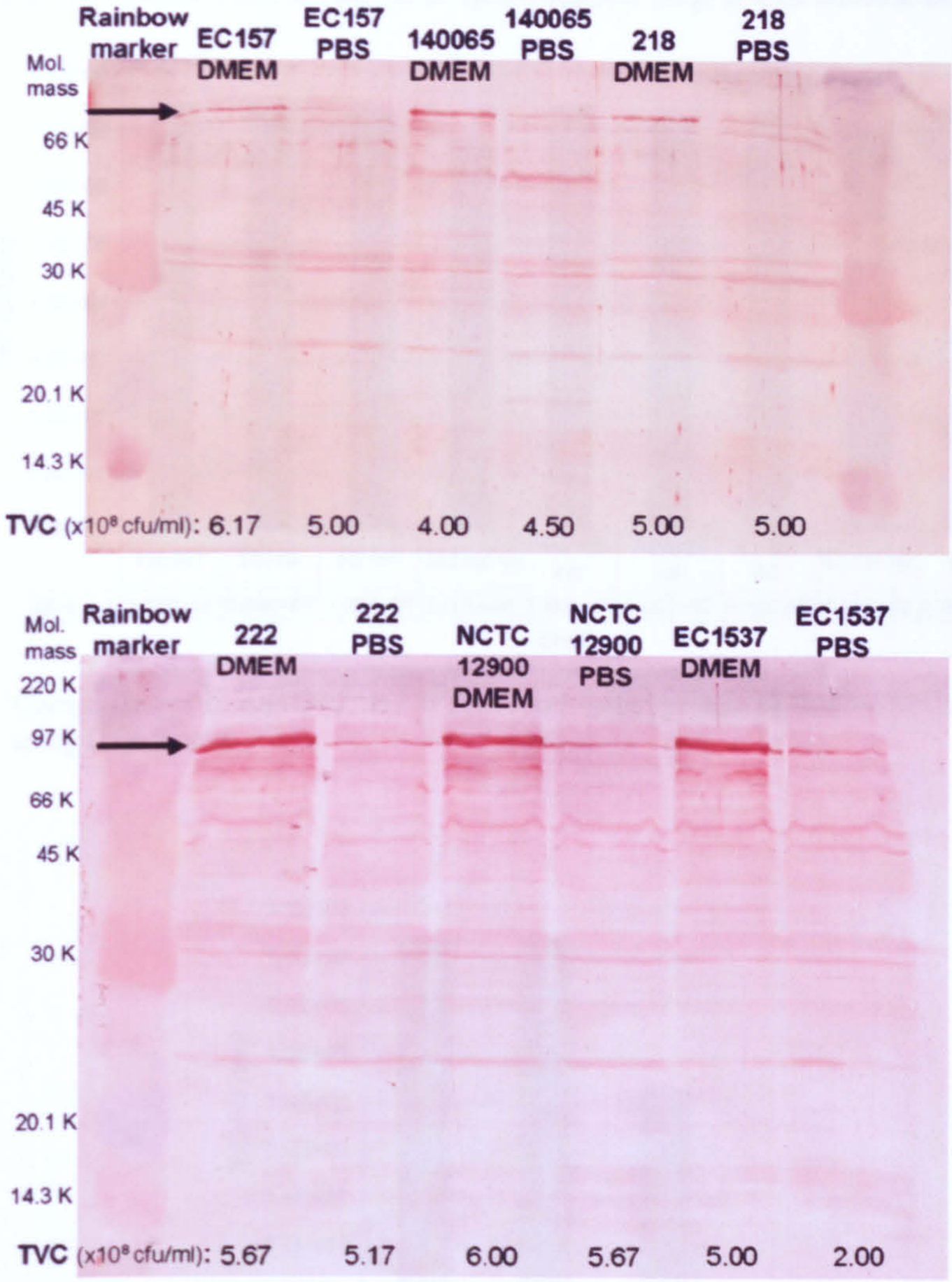
i. Silver (above) and Coomassie (right) -stained polyacrylamide gels of electrophoresed secreted proteins preparations. The lanes to the left of the divider above are from Figure 3-c.



ii. Western blot membranes of secreted protein preparations probed with $EspA_{O157}$ antibody. Double bands of approximately 20 kDa are present with three of the strains to the left and with two strains on the right (arrows). The lanes to the left of the divider are from Figure 3-c.

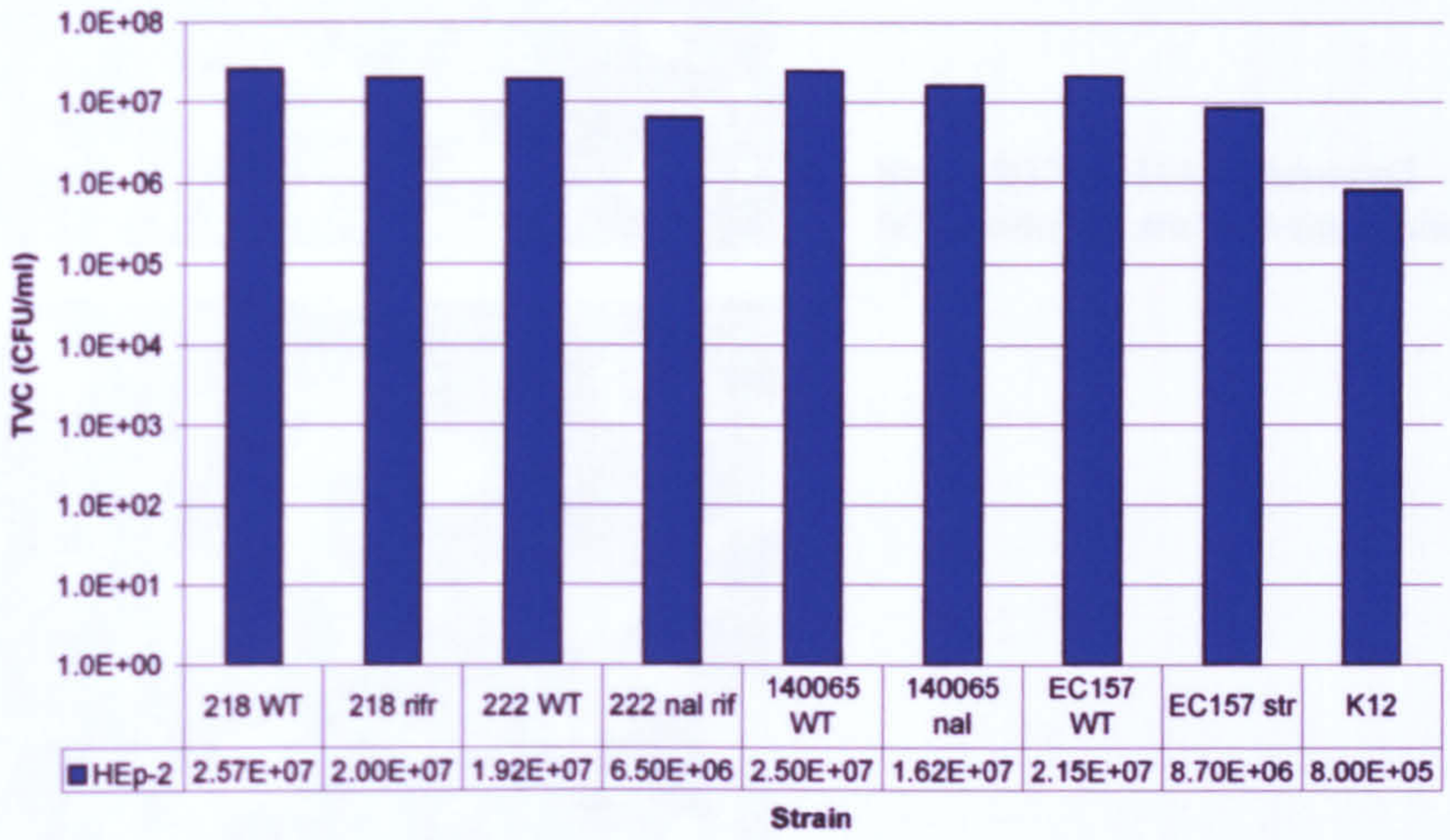
Notes: Strains were incubated either in DMEM at 37 °C for 5 hours, or in PBS at room temperature for one hour, before secreted proteins were concentrated by centrifugal ultrafiltration. The final TVC of each preparation after incubation is given at the bottom of each lane. Strain EC1537 (far right) is *E. coli* O26:H11; all others are *E. coli* O157:H7.

Figure 3-e: Western blots of *E. coli* cell lysates probed with γ-intimin antibody

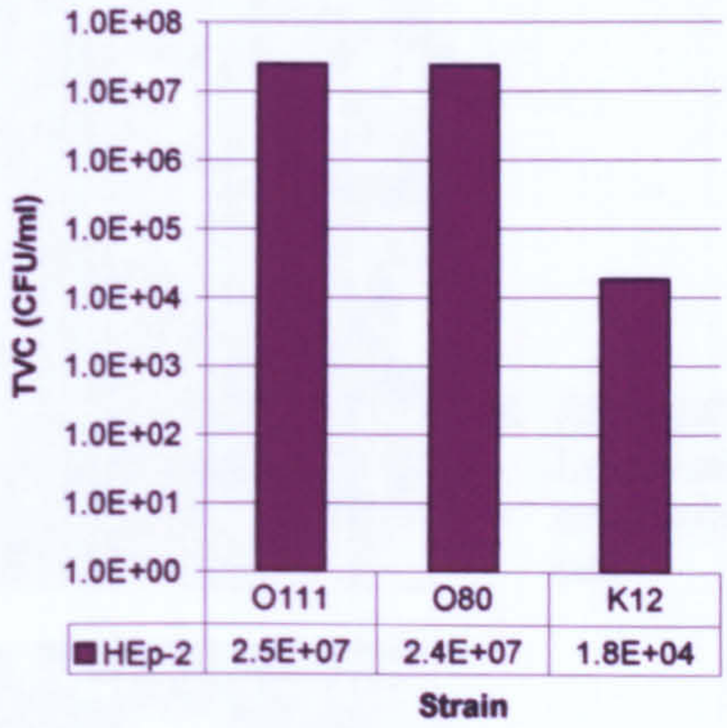


Notes: Strains were incubated either in DMEM at 37 °C for 5 hours, or in PBS at room temperature for one hour, before secreted proteins were concentrated by centrifugal ultrafiltration. The final TVC of each preparation after incubation is given at the bottom of each lane. Strain EC1537 (bottom right) is *E. coli* O26:H11; all others are *E. coli* O157:H7. The strong band corresponding with the 94-97 kDa $intimin_{O157}$ molecule is arrowed. Substantial non-specific band staining is present; this is probably due to the high dilution of primary antibody used.

Figure 3-f: Results from three-hour quantitative HEp-2 cell association assays

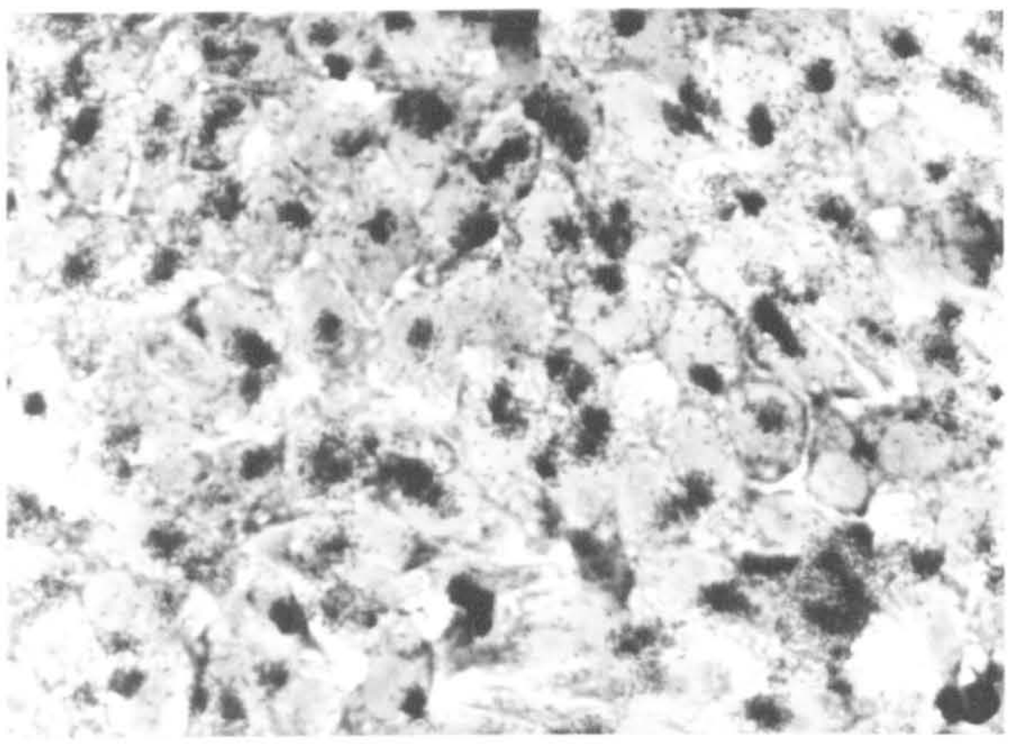


i. Comparison of *E. coli* O157:H7 WT and antibiotic resistant derivative strains using HEp-2 cells

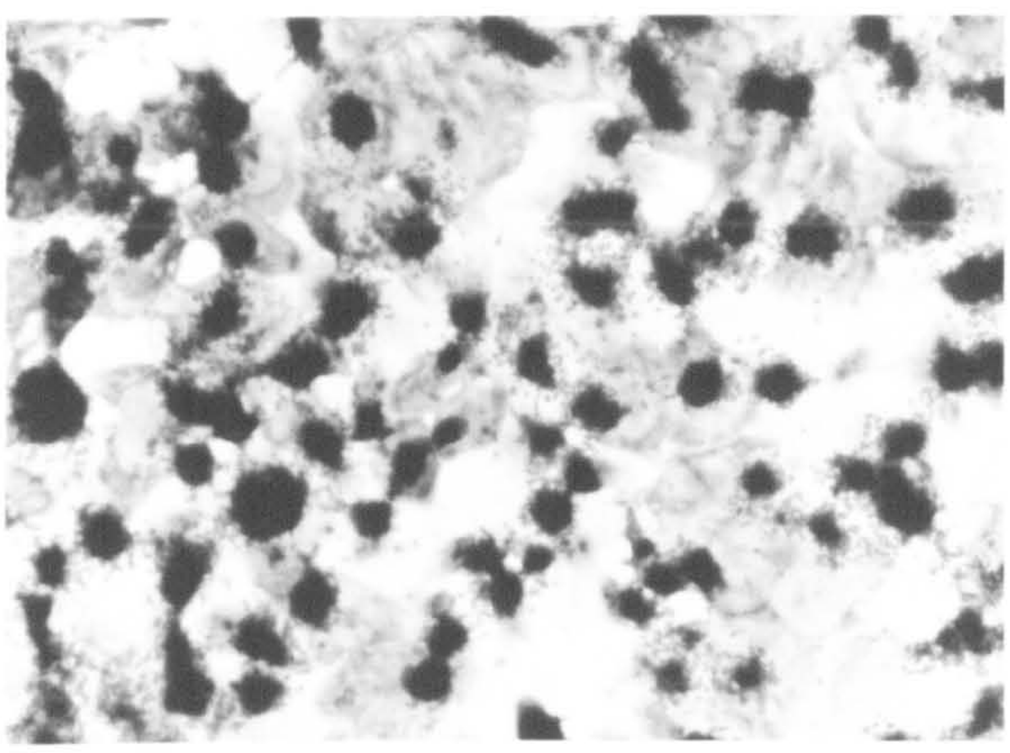


ii. Comparison of control strains B171 (O111) and 85440 (O80) using HEp-2 cells

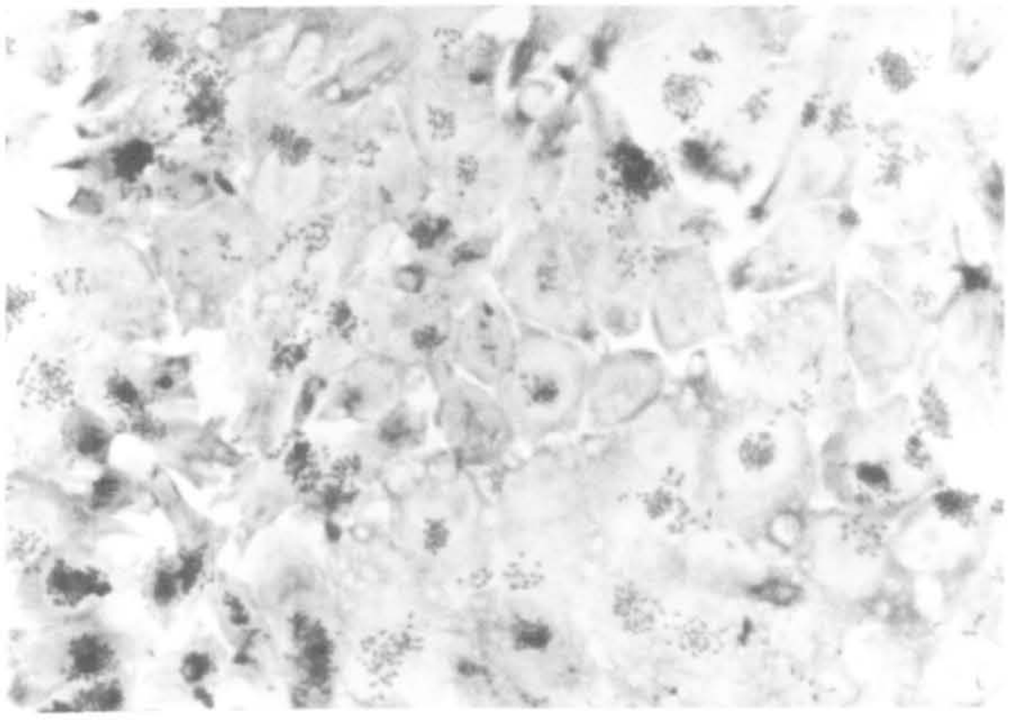
Figure 3-g: Giemsa-stained HEp-2 monolayers



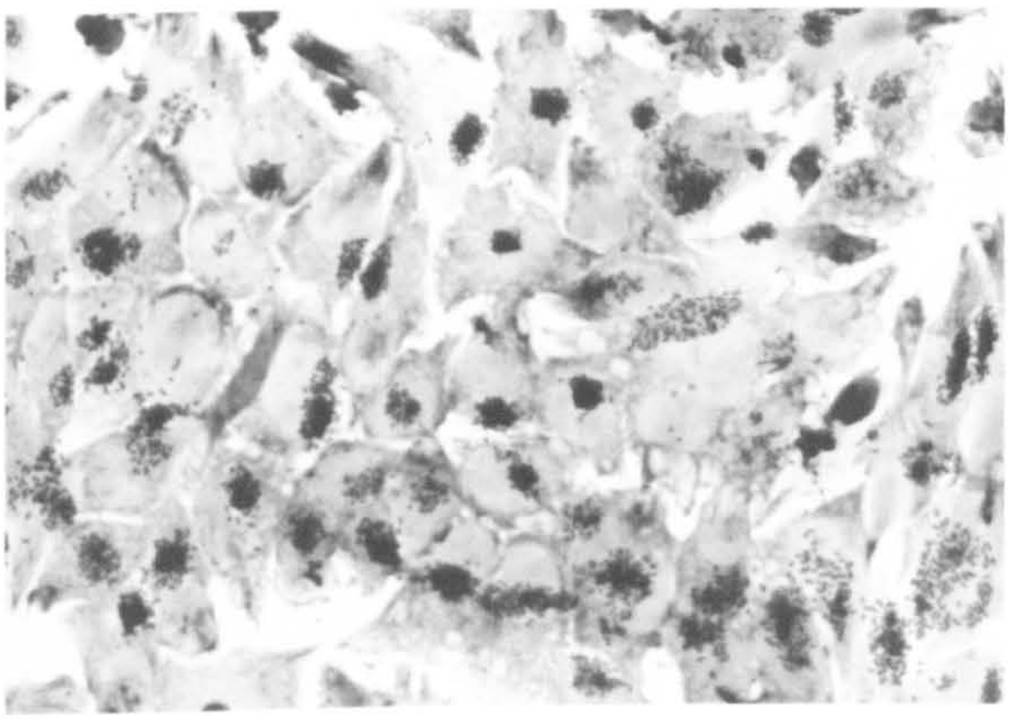
i. Strain B171(O111), 3 hours p.i. Microcolonies are well-established.



ii. Strain B171 (O111), 6 hours p.i. Large, dense microcolonies are present.

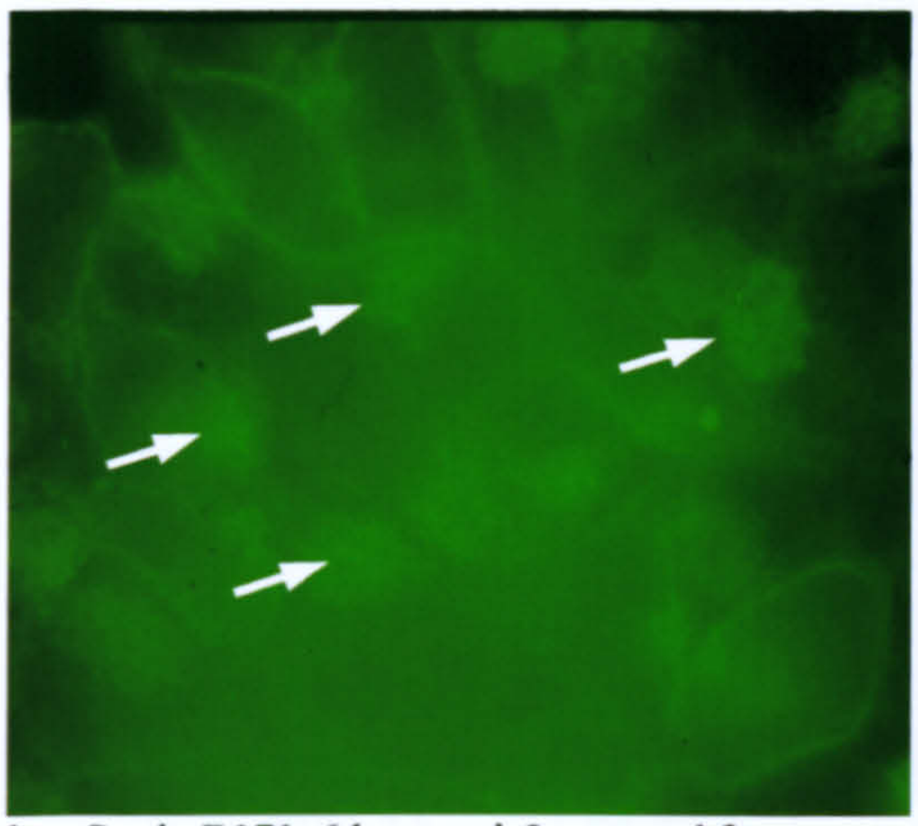


iii. Strain EC157 (O157), 6 hours p.i. Low to moderate density microcolonies are present on most cells.

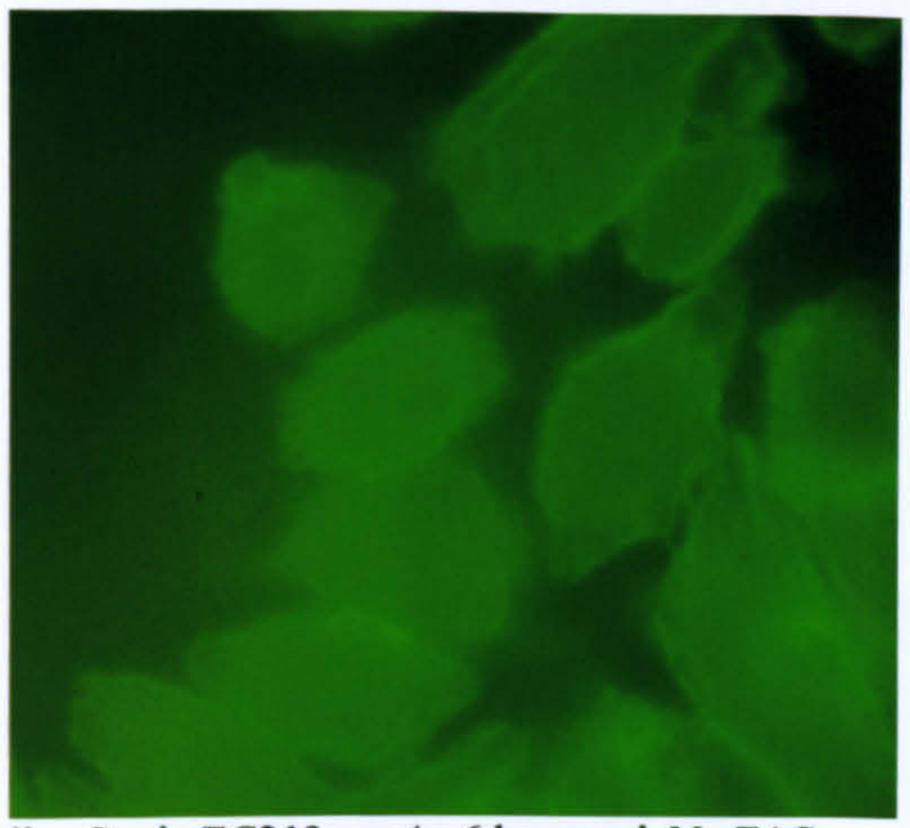


iv. Strain EC218 (O157), 6 hours p.i. Moderately dense microcolonies are present on most cells.

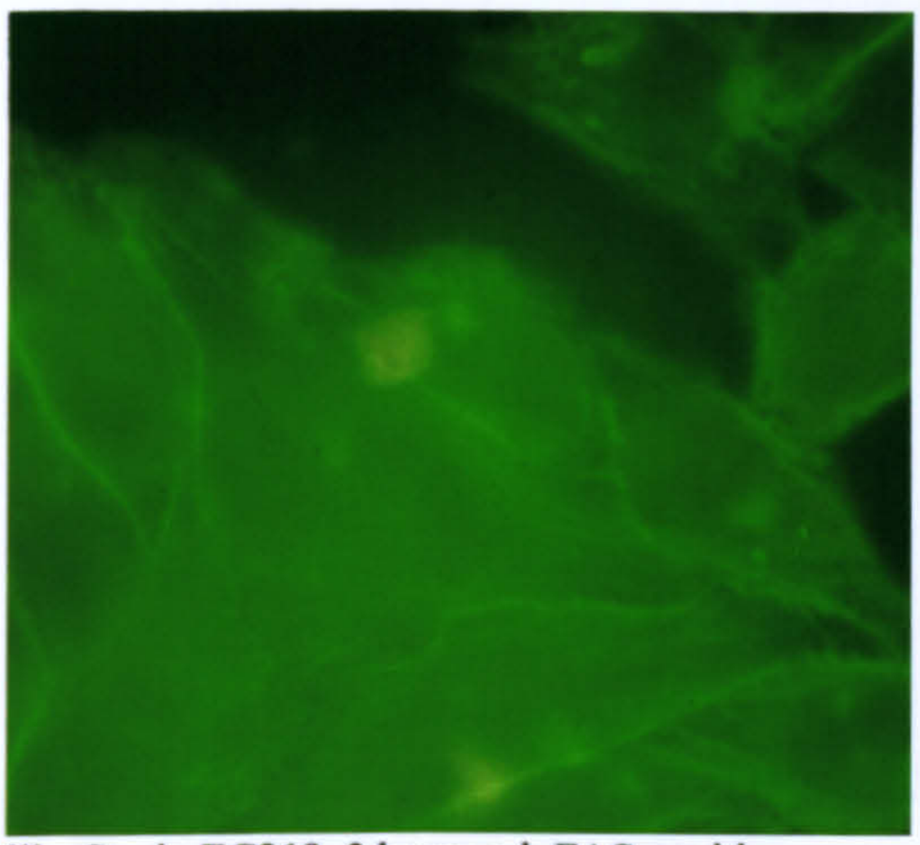
Figure 3-h: Fluorescence actin staining of HEp-2 monolayers inoculated with *E. coli*



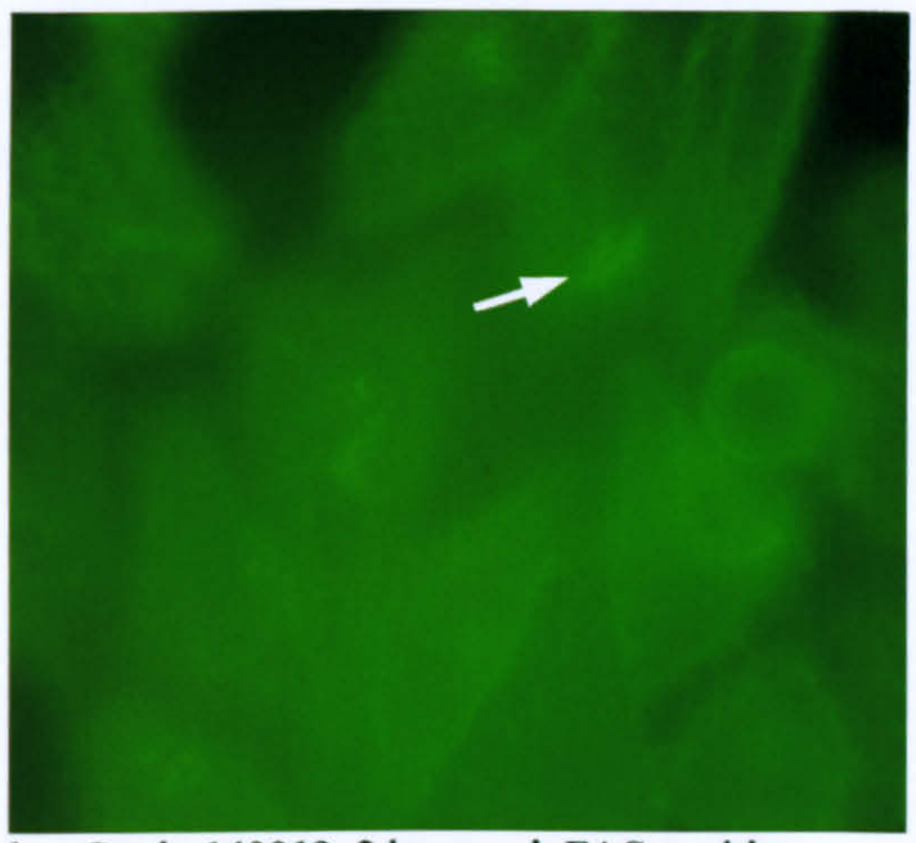
i. Strain B171, 6 hours p.i. Large and frequent FAS-positive colonies (examples arrowed).



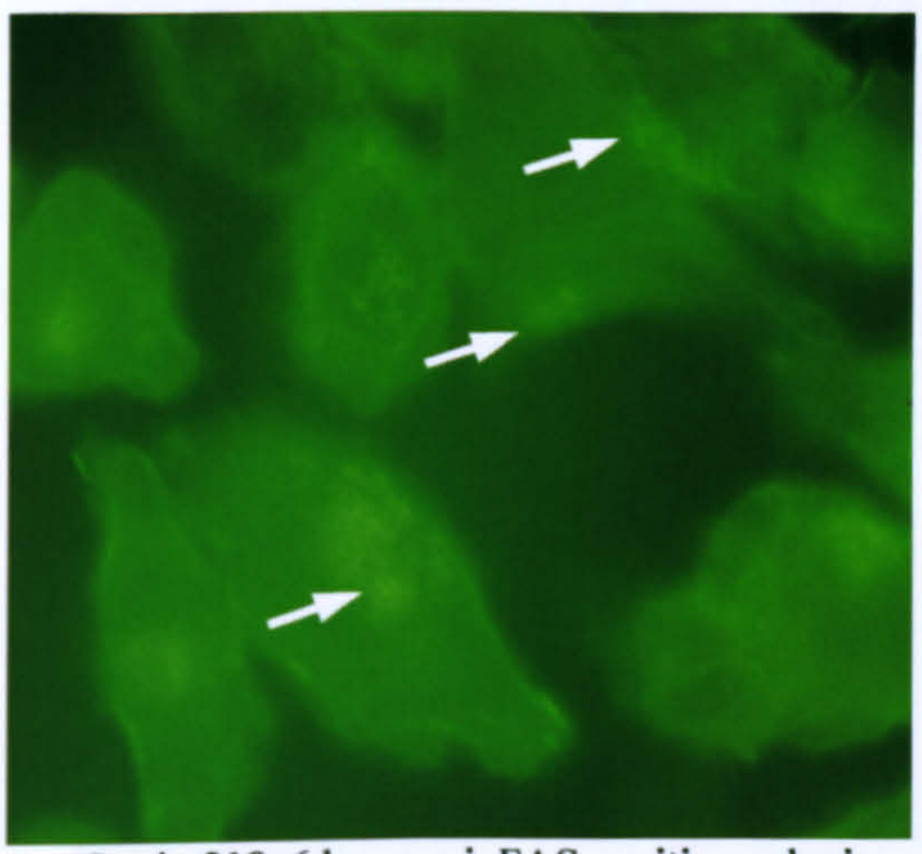
ii. Strain EC218 *eaeA*-, 6 hours p.i. No FAS-positive colonies.



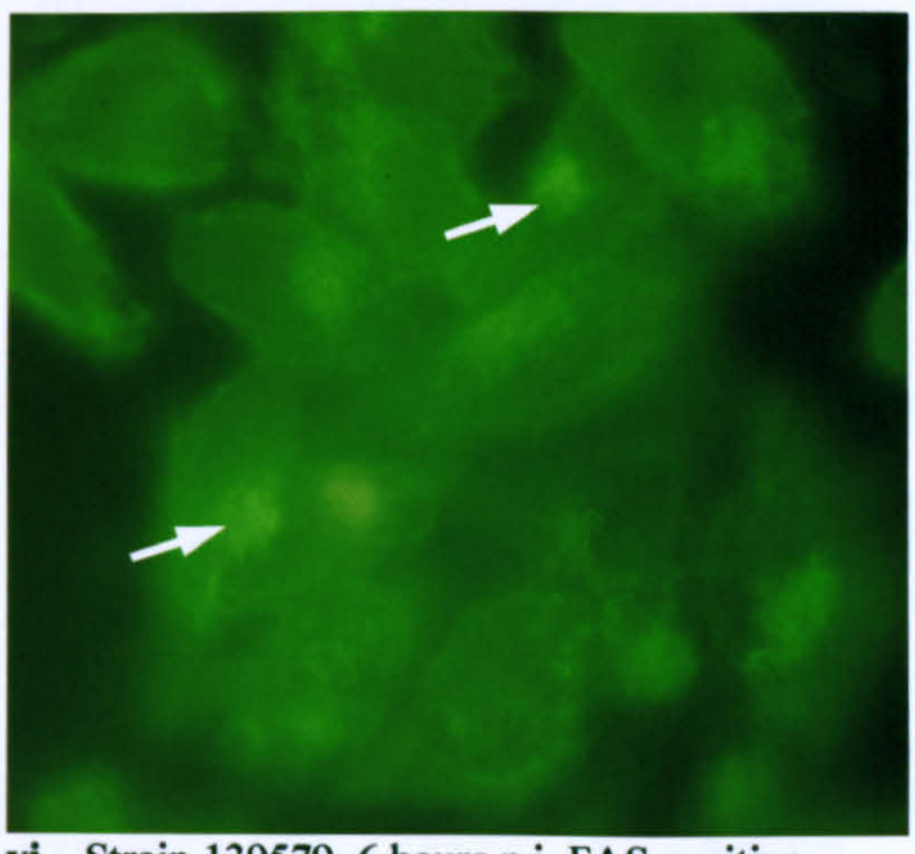
iii. Strain EC218, 3 hours p.i. FAS-positive colonies are not clearly evident.



iv. Strain 140019, 3 hours p.i. FAS-positive colonies (arrowed) are small and scattered.

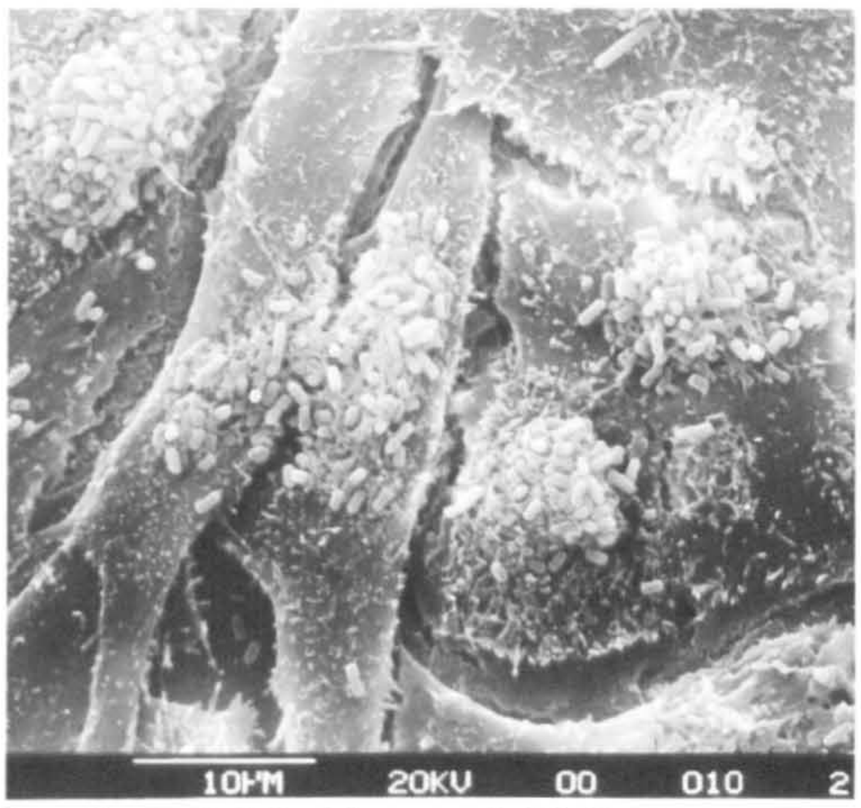


v. Strain 218, 6 hours p.i. FAS-positive colonies (examples arrowed) are of moderate size and are common.

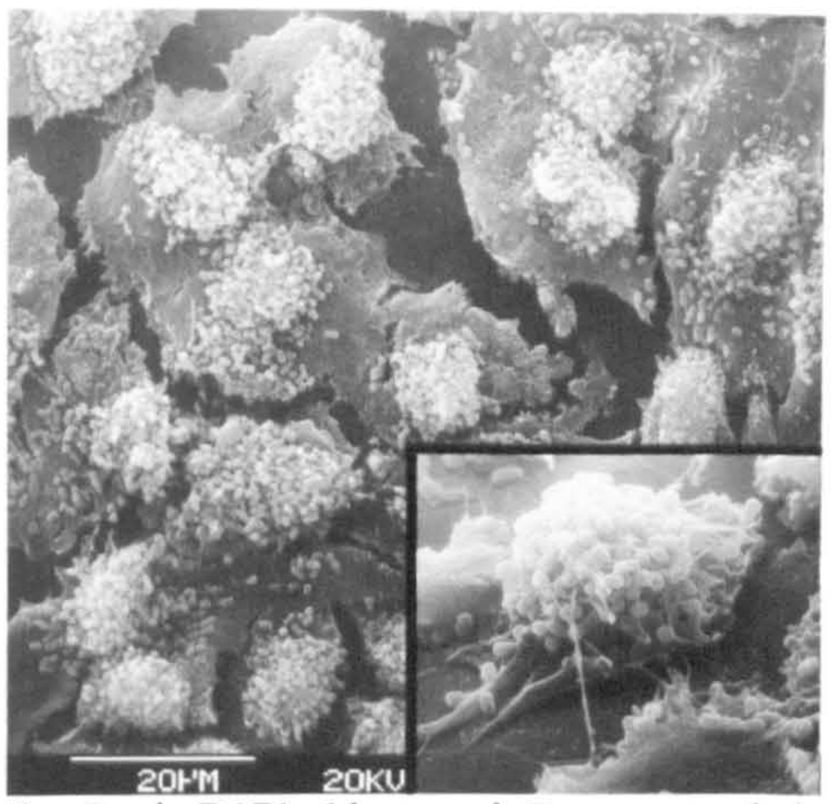


vi. Strain 139579, 6 hours p.i. FAS-positive colonies (examples arrowed) are of moderate size.

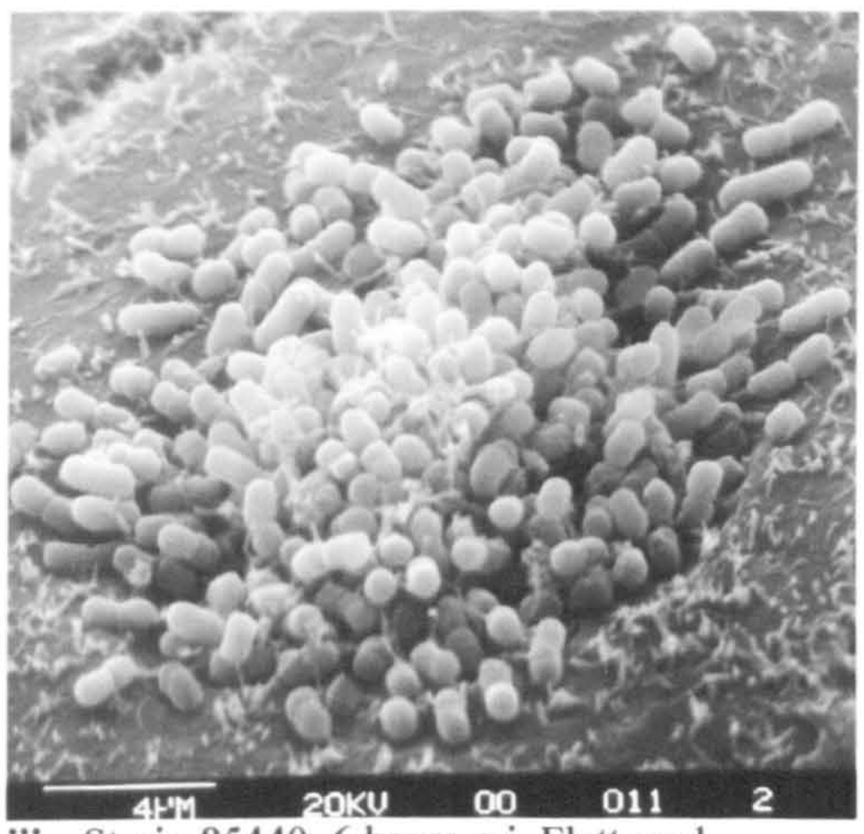
Figure 3-i: Scanning electron micrographs of HEp-2 monolayers inoculated with *E. coli*



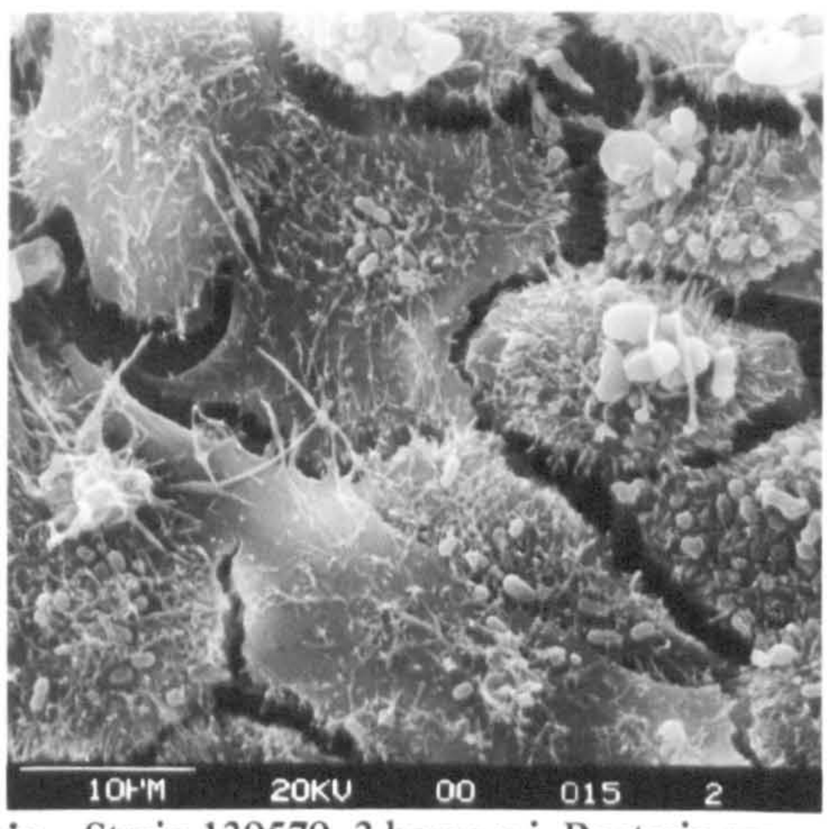
i. Strain B171, 3 hours p.i. Distinct microcolonies.



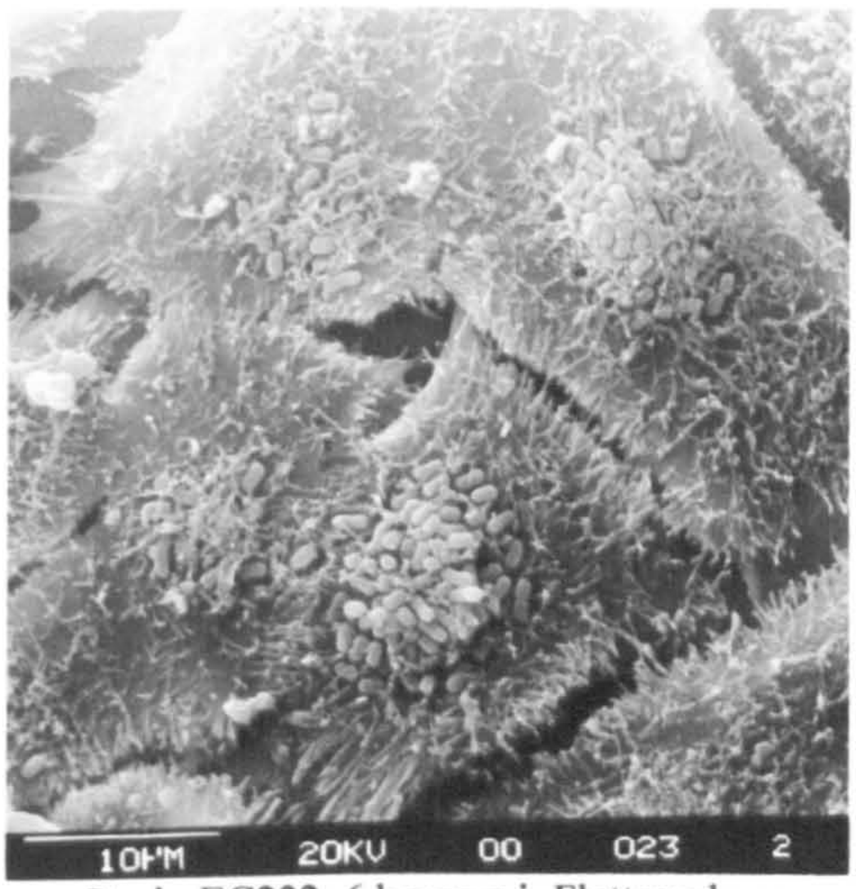
ii. Strain B171, 6 hours p.i. Dense, rounded (inset) microcolonies on all cells.



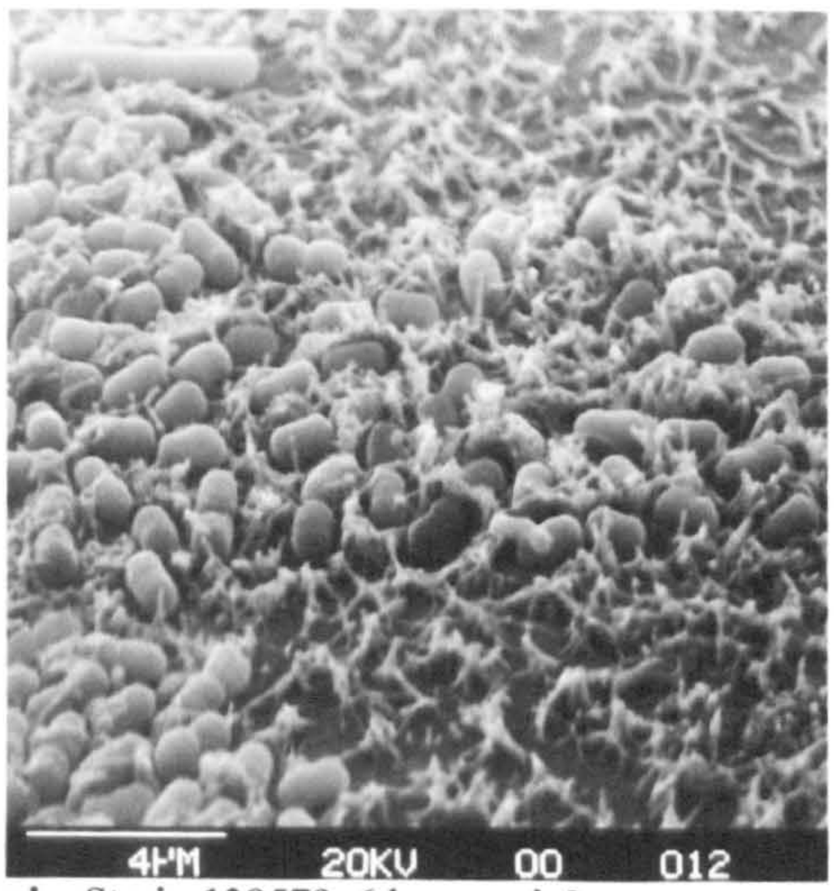
iii. Strain 85440, 6 hours p.i. Flattened, moderately dense microcolony.



iv. Strain 139579, 3 hours p.i. Bacteria are scattered, some small clusters discernible.

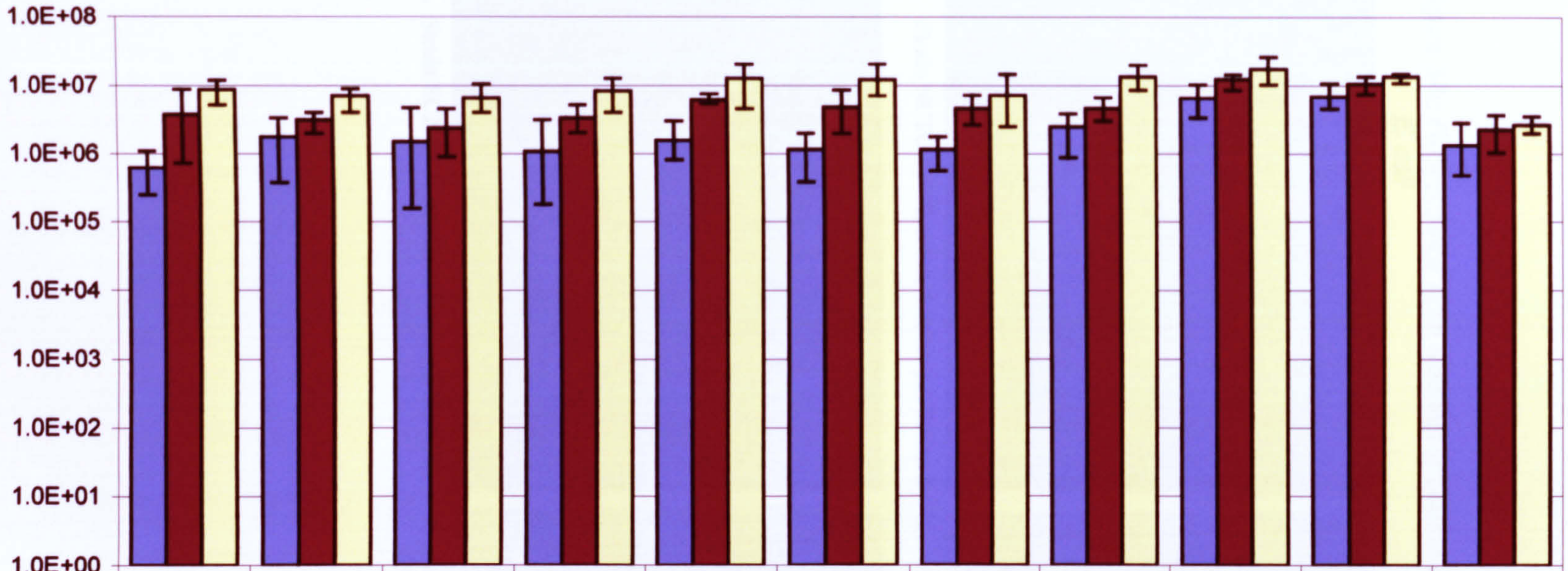


v. Strain EC222, 6 hours p.i. Flattened microcolonies of varying density on most cells.



vi. Strain 139579, 6 hours p.i. Loose aggregates of bacteria.

Figure 3-j: Results of *E. coli* - bovine intestinal cell culture quantitative association assays

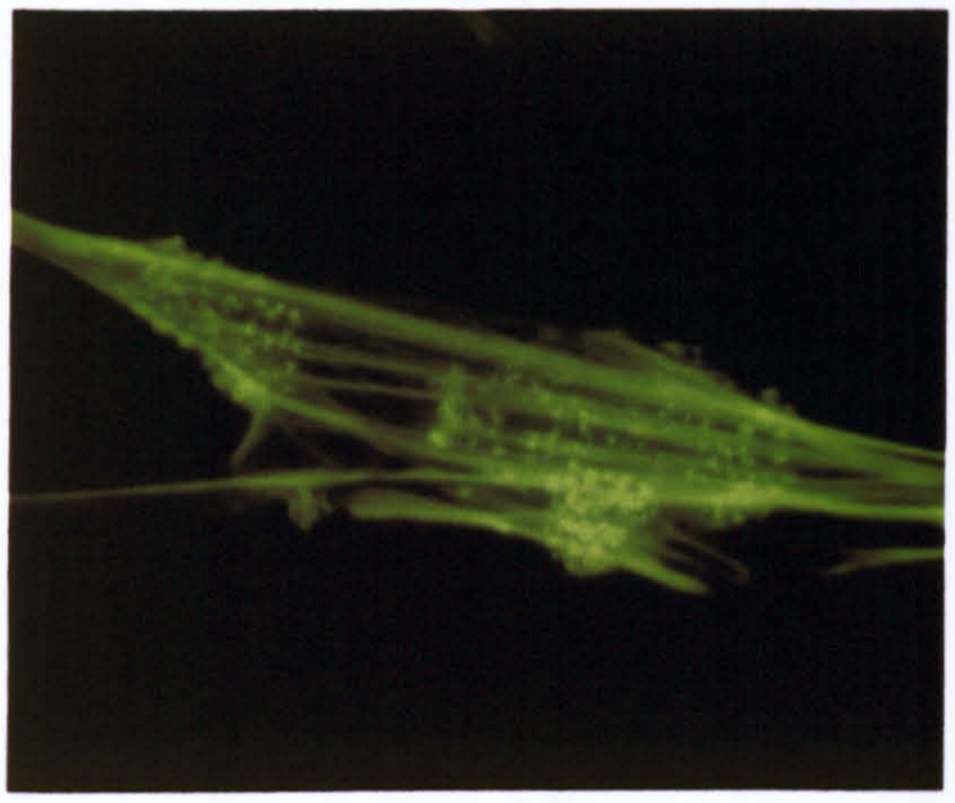


	140065 WT	140065 nalr	EC157 WT	EC157 strr	218 WT	218 rifr	222 WT	222 nalrrifr	NCTC 12900	EC1537 (026)	DH5α
Ileum	6.2E+05	1.8E+06	1.5E+06	1.1E+06	1.6E+06	1.1E+06	1.1E+06	2.5E+06	6.6E+06	6.9E+06	1.3E+06
Colon	3.8E+06	3.2E+06	2.4E+06	3.3E+06	6.1E+06	4.8E+06	4.5E+06	4.6E+06	1.2E+07	1.1E+07	2.3E+06
Rectum	8.6E+06	7.0E+06	6.5E+06	8.3E+06	1.3E+07	1.2E+07	7.2E+06	1.3E+07	1.7E+07	1.4E+07	2.7E+06

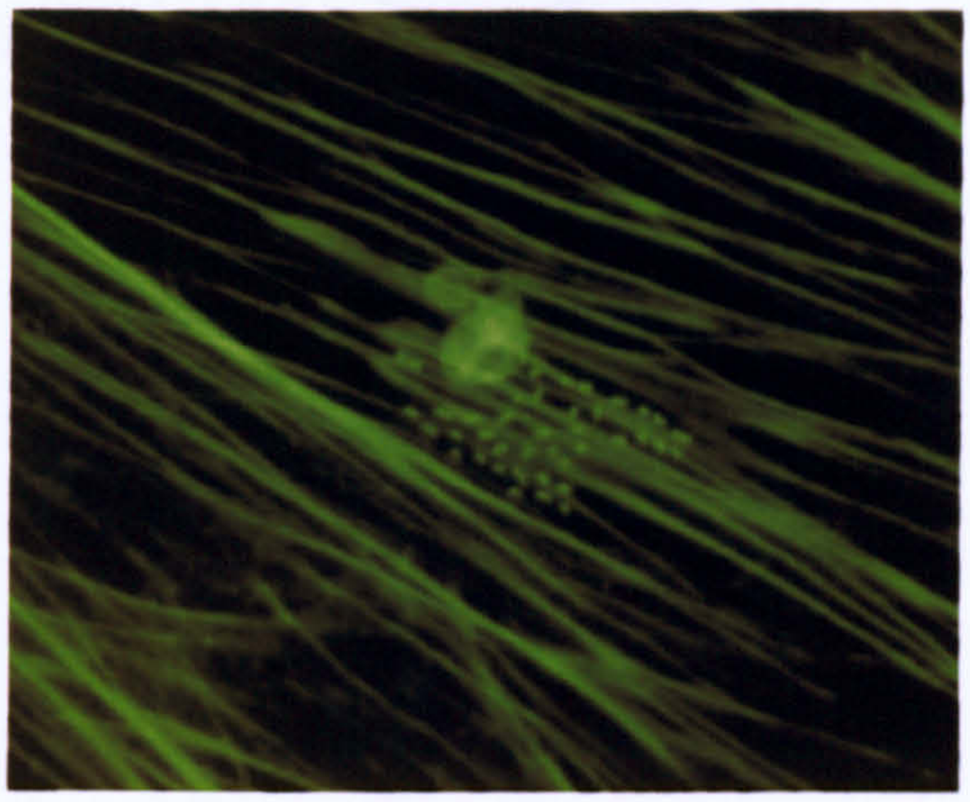
Strains

The chart shows the mean plus range of four assays per strain-cell combination. WT = wild-type. DH5α is a K12 laboratory strain.

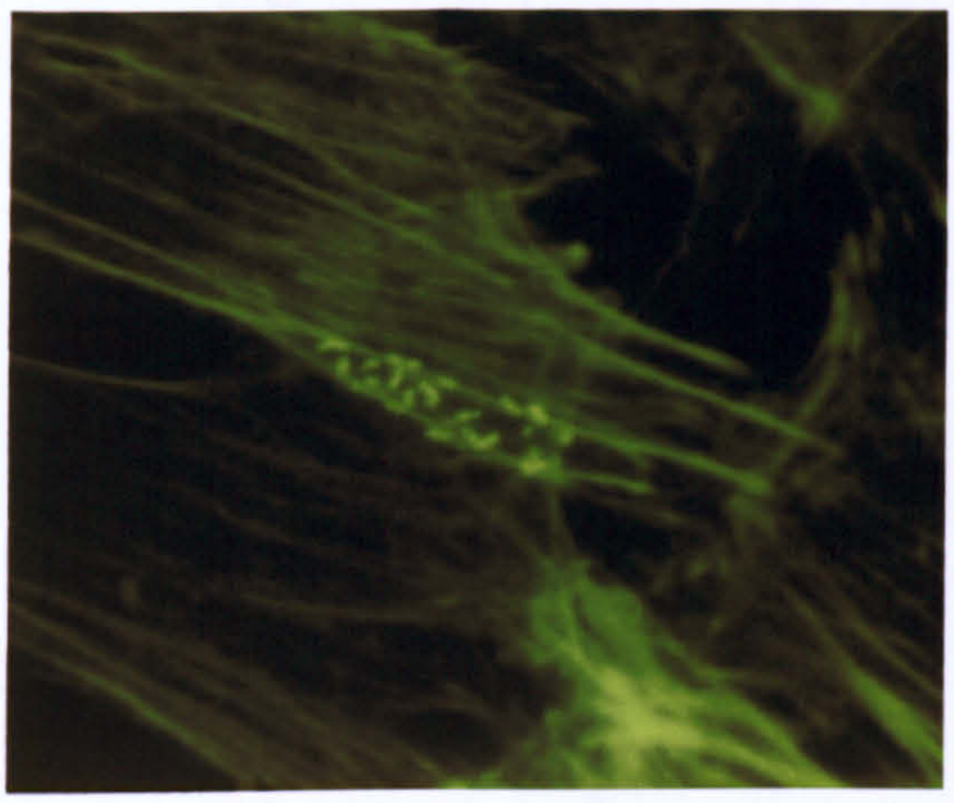
Figure 3-k: Fluorescence actin staining of bovine intestinal cell cultures inoculated with *E. coli*



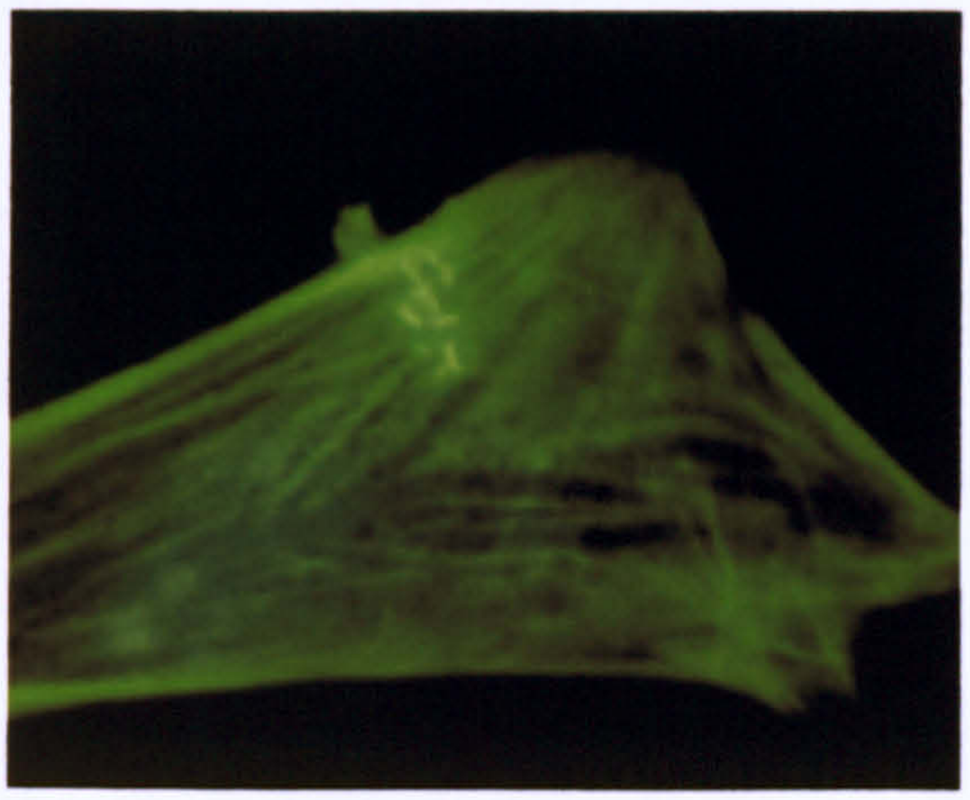
i. Strain NCTC 12900, colon, 6 hours p.i. Score: 5



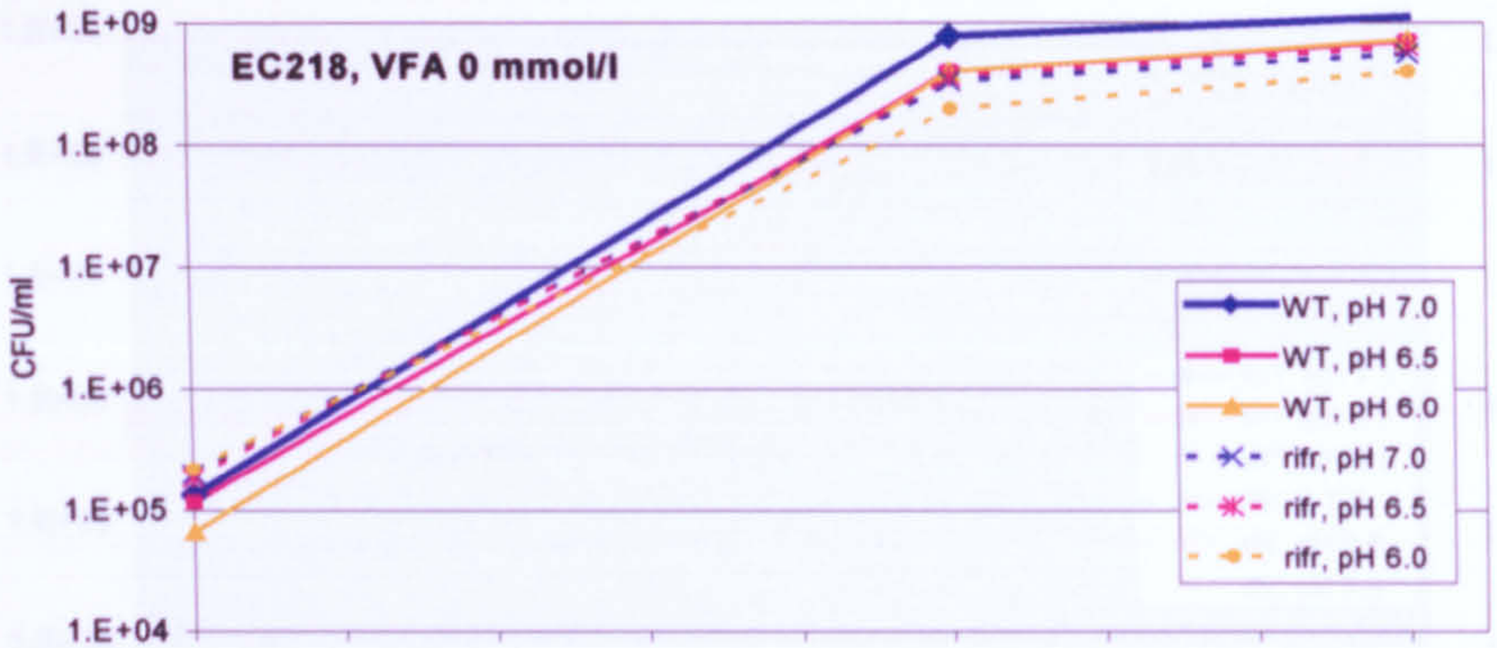
ii. Strain EC1537, ileum, 6 hours p.i. Score: 2.

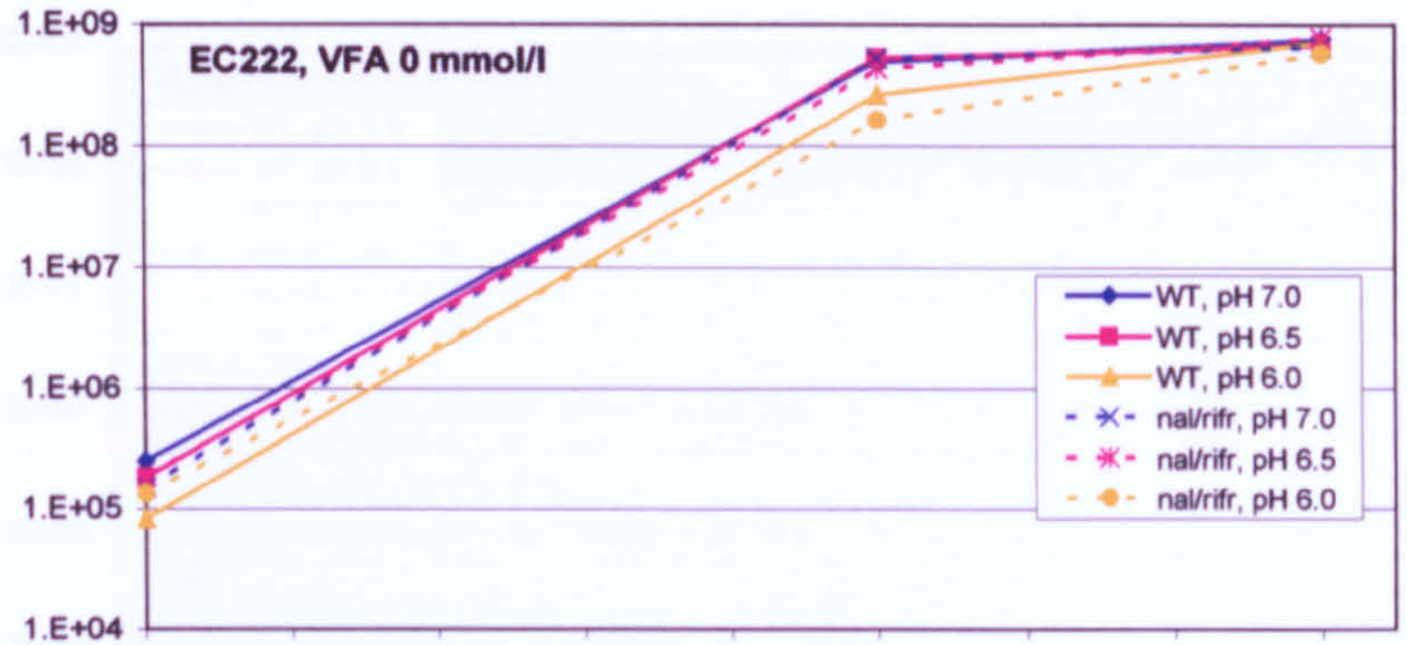


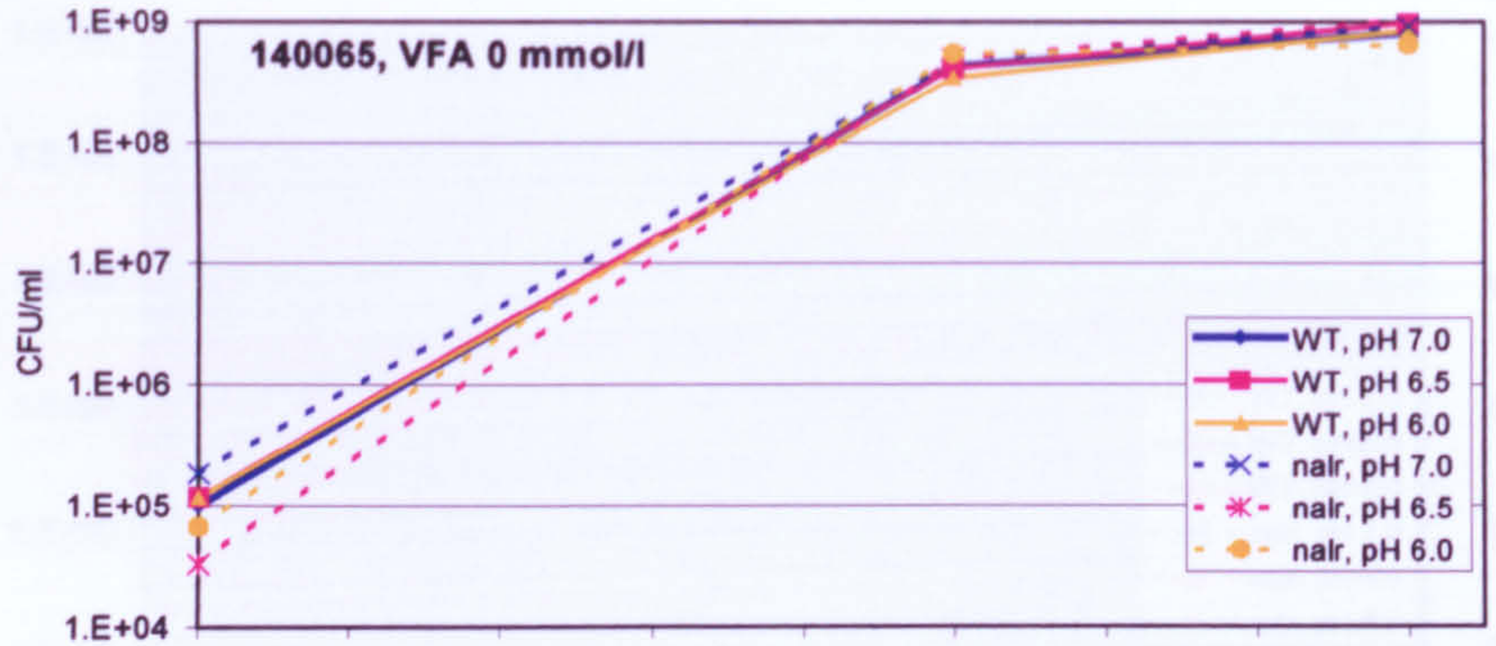
iii. Strain EC218 rif^r, rectum, 6 hours p.i. Score: 2.

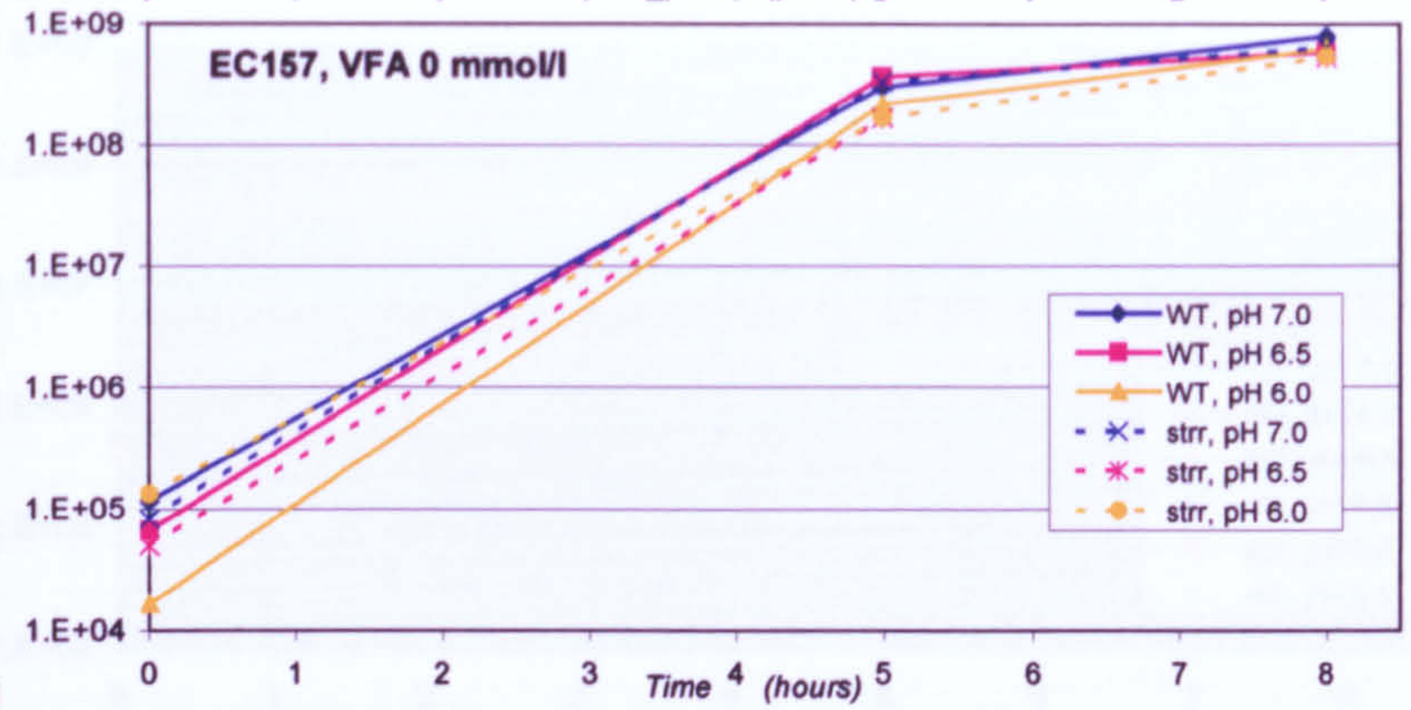


iv. Strain EC222 $nal^r rif^r$, rectum, 6 hours p.i. Score: 1.









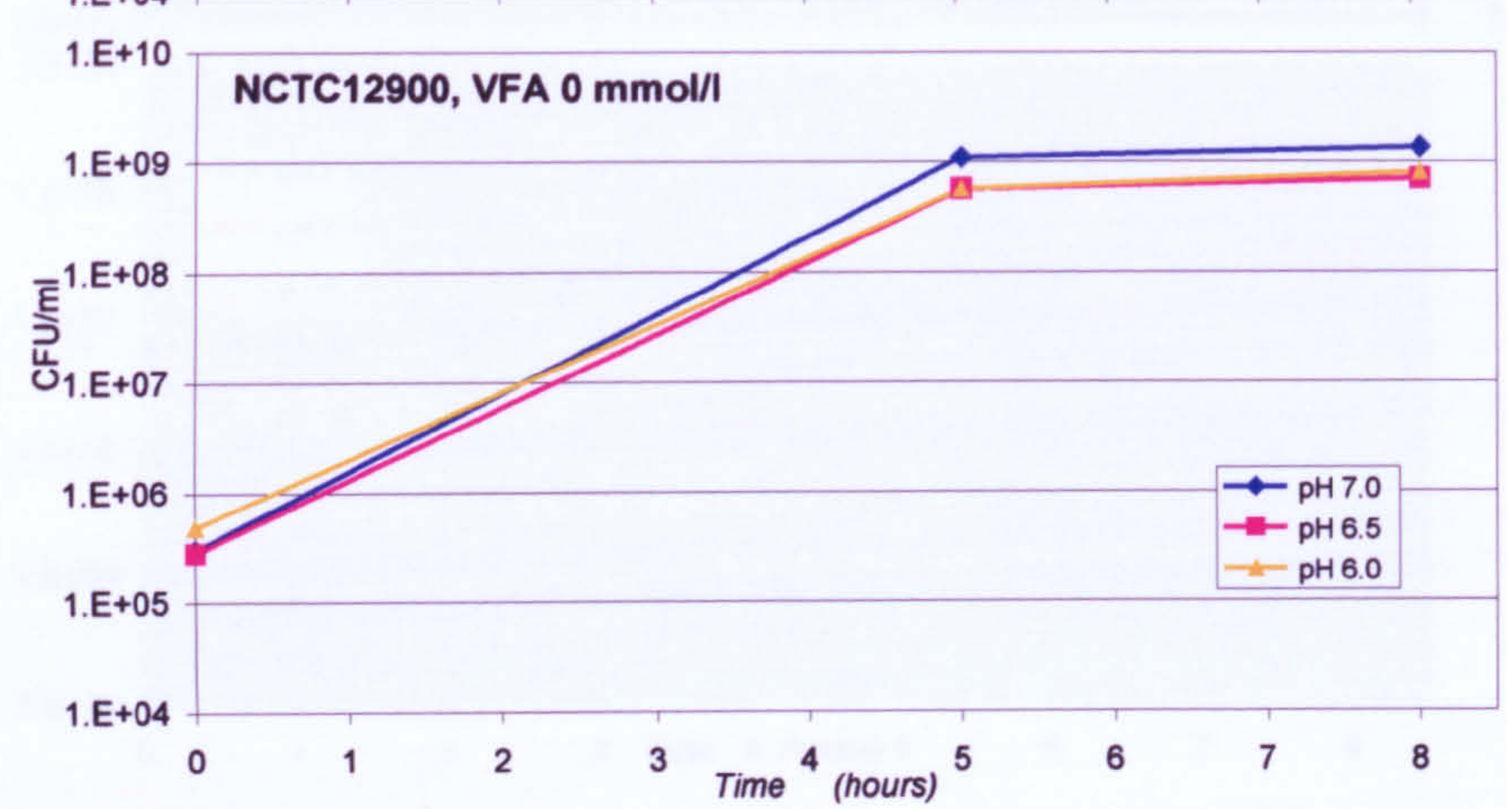
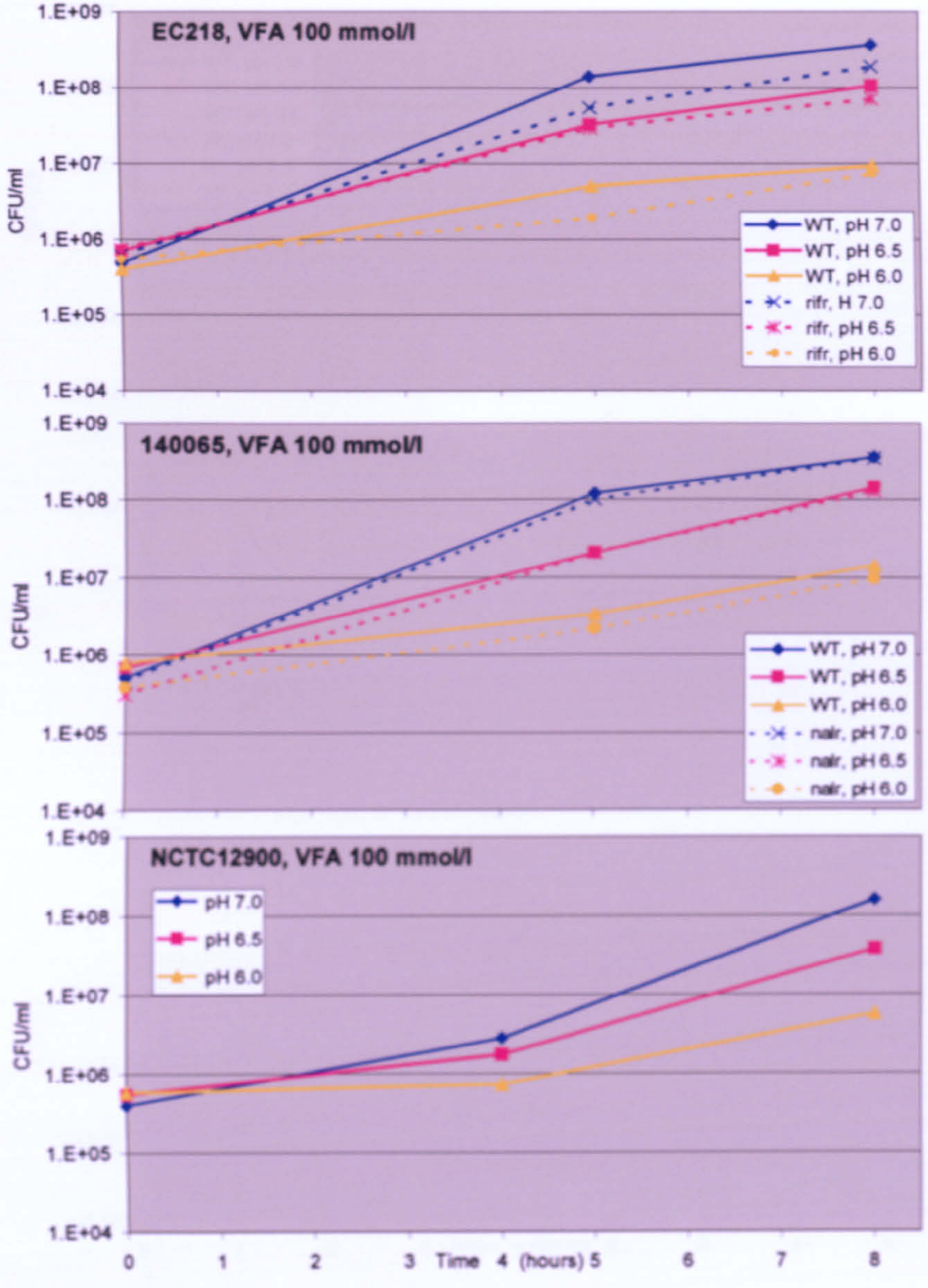


Figure 3-I: Growth of *E. coli* O157:H7 with VFA (1)

LB-G broth, no added VFA.
Incubated at 37°C anaerobically.



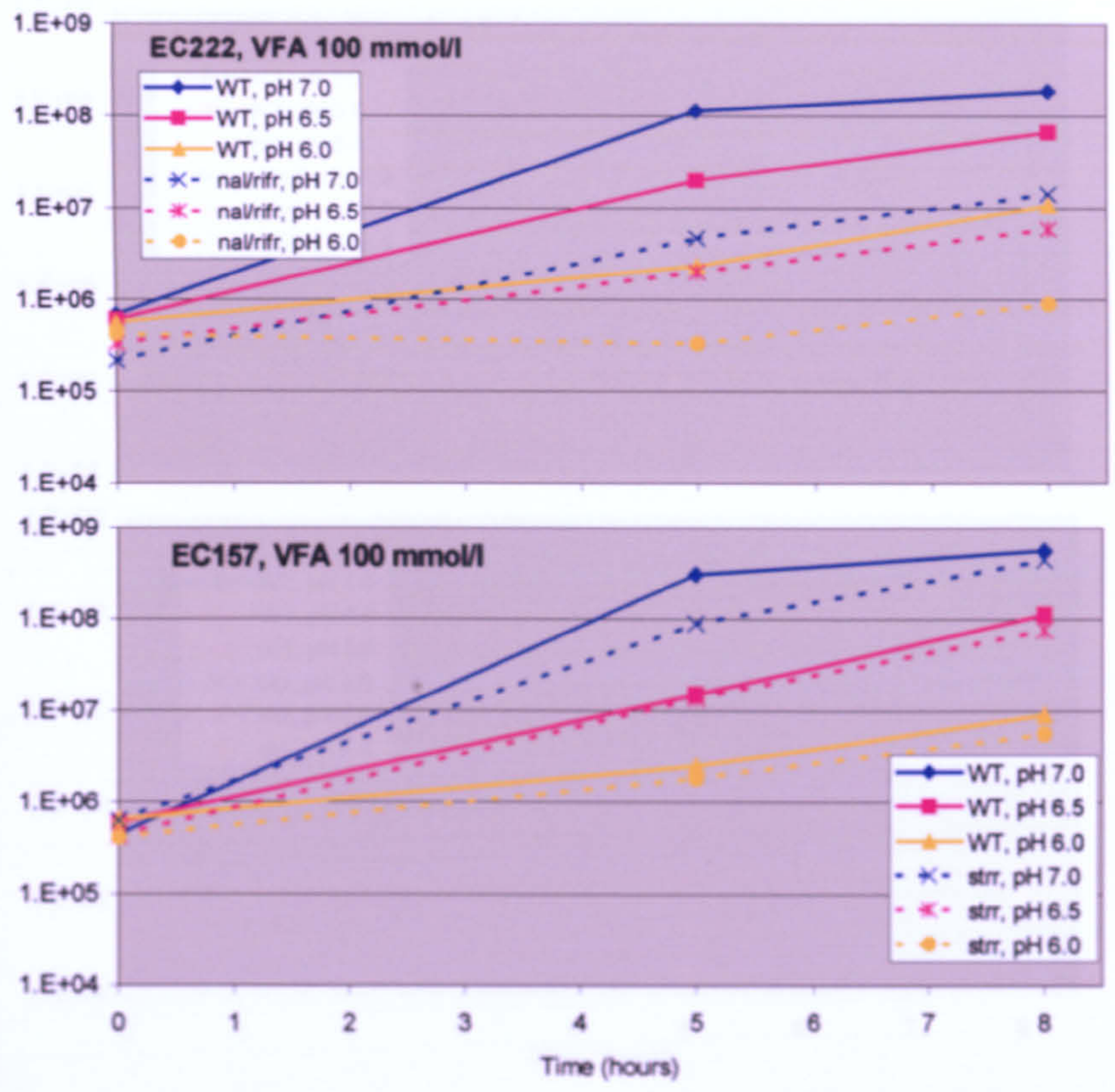


Figure 3-m: Growth of *E. coli* O157:H7 with VFA (2)

LB-G broth, added VFA to 100 mmol/l.
Molar ratio: Acetate 75 %, Propionate 10 %,
Butyrate 10 %, Valerate 5 %.
Incubated at 37 °C anaerobically.

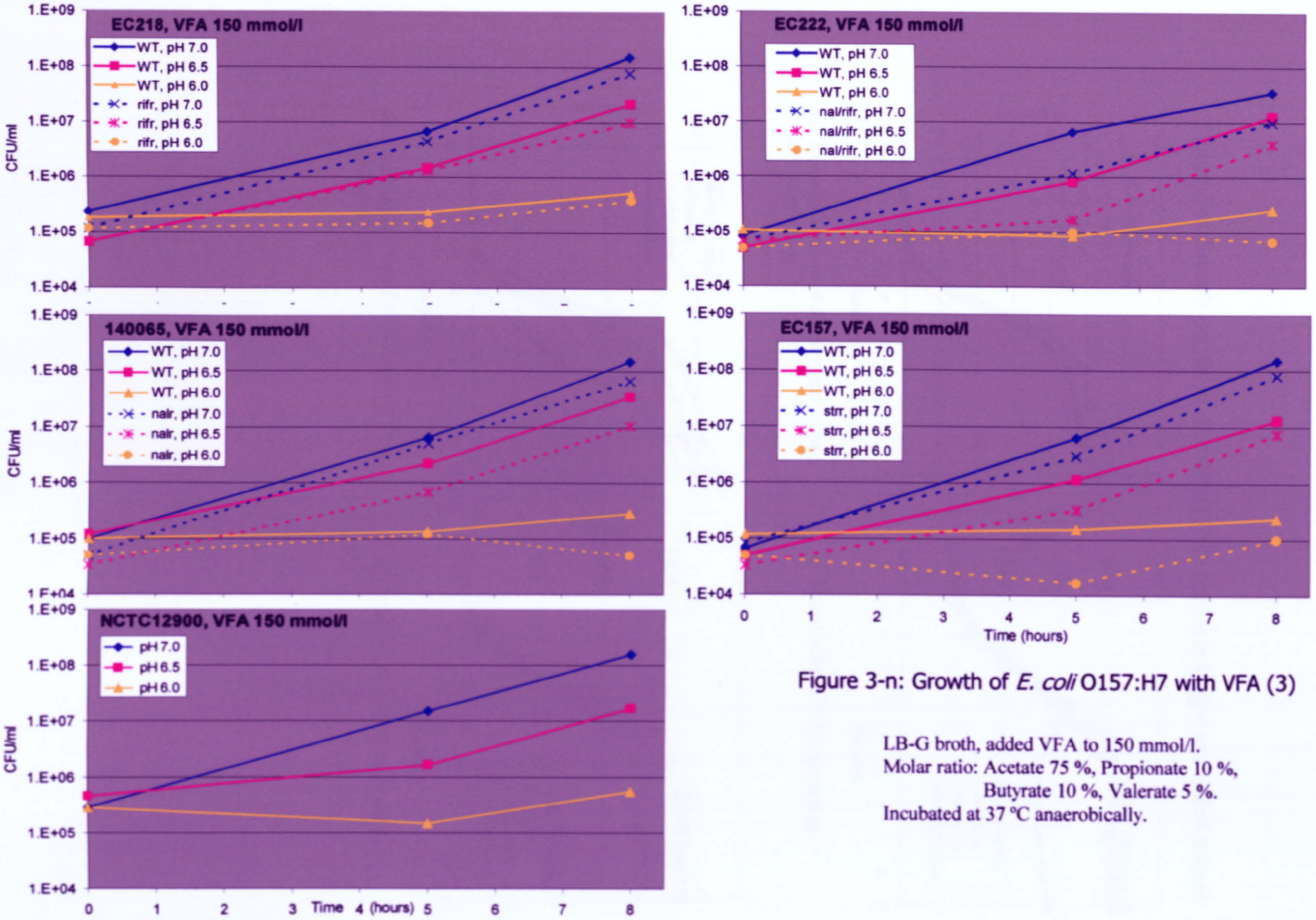
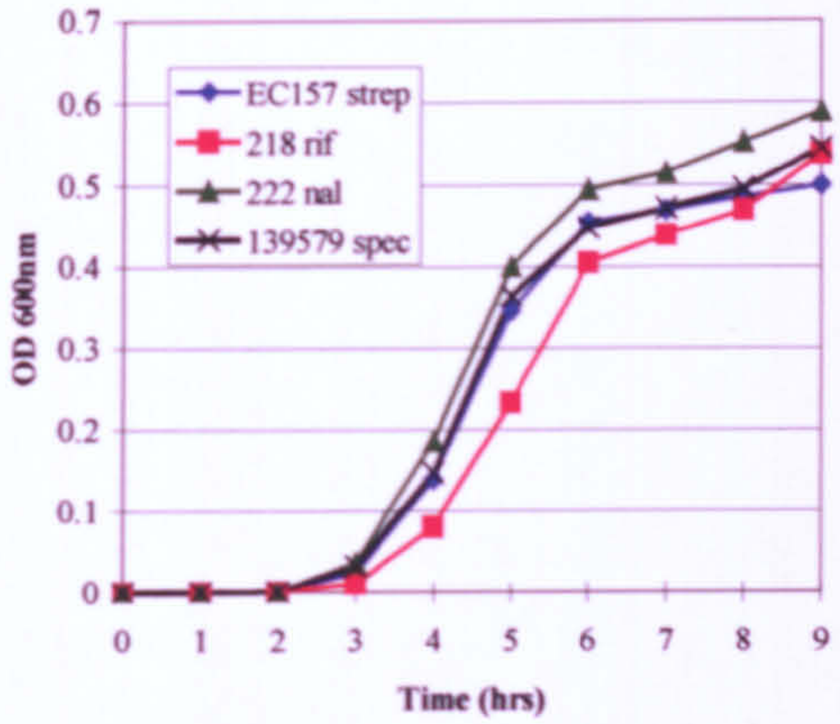


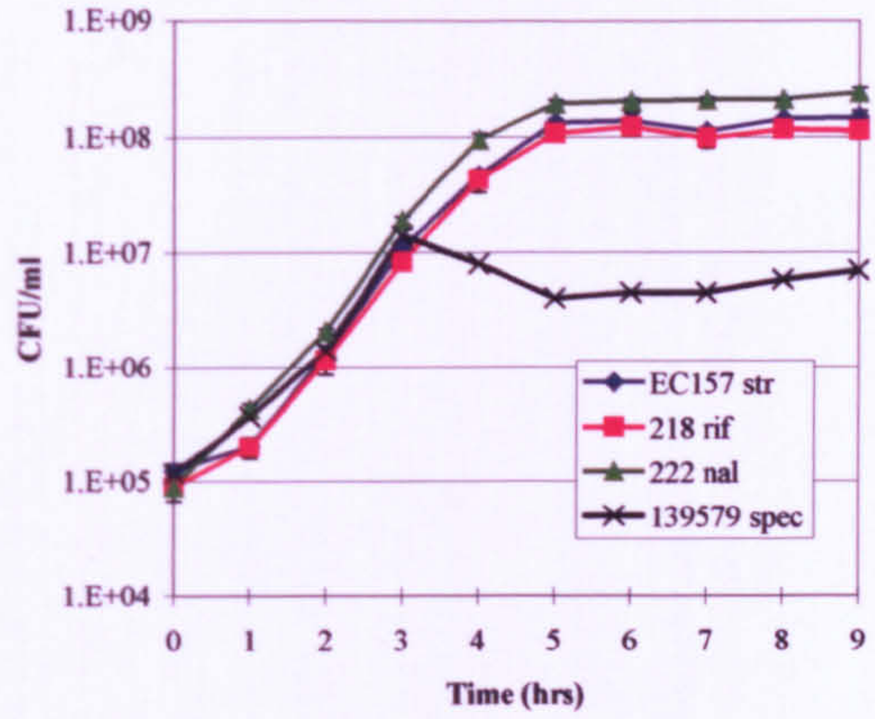
Figure 3-n: Growth of *E. coli* O157:H7 with VFA (3)

LB-G broth, added VFA to 150 mmol/l.
Molar ratio: Acetate 75 %, Propionate 10 %,
Butyrate 10 %, Valerate 5 %.
Incubated at 37 ℃ anaerobically.

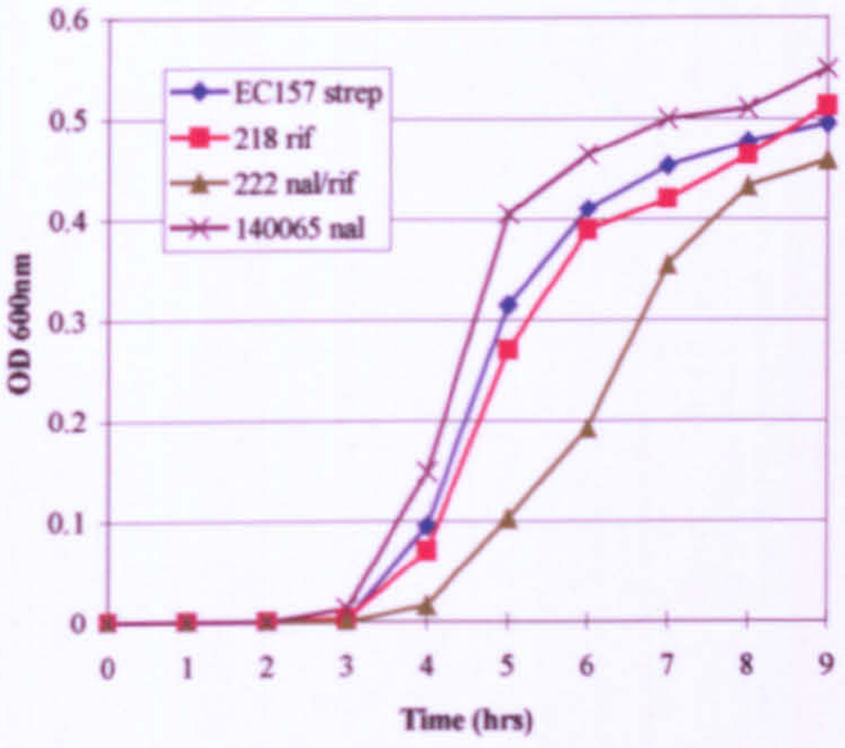
Figure 3-o: Growth curves of *E. coli* O157:H7 strains in single and mixed culture



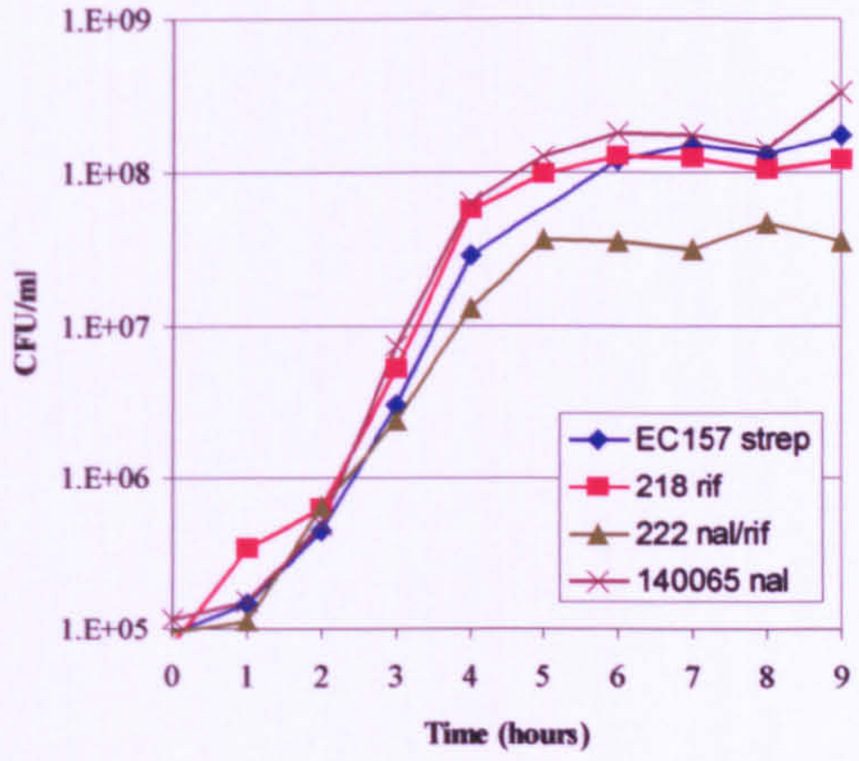
i. Combined curves of single strain culture, OD monitoring.



ii. Mixed culture, TVC monitoring.



iii. Combined curves of single strain culture, OD monitoring.



iv. Mixed culture, TVC monitoring.

Chapter 4 – INTESTINAL LOOP STUDIES

4.1 Introduction

The occurrence of persistent excretion of *E. coli* O157:H7 by some ruminants has been documented (Besser et al. 1997; Kudva et al. 1997a; Mechie et al. 1997; Shere et al. 1998). Adherence of the bacterium to the intestinal wall *in vivo* has been reported (since the completion of the present studies) in a naturally colonised steer at the recto-anal junction (Naylor et al. 2003). Experimentally, a high dose (10^9 to 10^{10} CFU) oral inoculum of *E. coli* O157:H7 has yielded detectable AE lesions in the large intestine of neonatal and weaned calves (Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1998; Dean-Nystrom et al. 1999) and in neonatal lambs (Chapter 5), although not in the context of persistent colonisation. It is possible that small, sparse and as-yet undetected AE lesions occur in naturally colonised animals and contribute to persistence. The ligated intestinal loop technique (Williams Smith and Halls 1967; Frost et al. 1997) is of potential use in investigating the adherence of *E. coli* O157:H7 to the ruminant intestinal mucosa. It facilitates the search for bacterial attachment by providing a well-defined mucosal area which has been subjected to a high concentration of inoculated bacteria. This may be used to establish the principle of susceptibility to AE lesions for a host species and for a particular age group. Other benefits include the ability to screen several bacterial strains for host interactions in a single animal, the potential to analyse bacterial secretions in a single, isolated loop and the potential to examine local host responses.

For the reasons discussed above, it was decided to use the ligated intestinal loop technique in the investigation of interactions between *E. coli* O157:H7 and the ruminant host. Sheep were used as the experimental species because they exhibit the phenomenon of natural persistent colonisation by *E. coli* O157:H7 in the field (Kudva et al. 1996; Kudva et al. 1997a), are associated with human infection (Licence et al. 2001; Strachan et al. 2001) and they are of convenient size for studies across a wide age range. The aims of the present study was: to establish effective methods for the use of this technique under hazard category 3 microbiological containment, and to examine further the adherence phenotype of the *E. coli* O157:H7 strains used in oral inoculation studies. The use of weaned lambs between five and six months old provided the opportunity to examine *E. coli* O157:H7 adherence in an age group in which the persistent excretion of the organism has been demonstrated (Section 1.4.2.7).

Four experiments were performed. The first (using Lamb 1) was a technical trial using sterile inocula to test, and modify if necessary, the methods which were based upon bovine (Frost et al. 1997) and ovine (Williams Smith and Halls 1967) small intestine loop studies,

and also to examine the effects of the manipulations upon the gross and histological appearance of the intestine. The second (using Lamb 2) was performed with category 2 AEEC, to further refine the technique and to examine the adherence phenotype of the two control non-O157 organisms plus one non Shiga toxin-producing *E. coli* O157:H7 which were used. The final two experiments (using lambs 3 and 4) used category 2 and 3 *E. coli* O157:H7 strains, these being the strains used in the oral inoculation studies (chapters 5 and 6), plus a category 3 natural bovine-pathogenic *E. coli* (O26:H11). This last organism was used as a comparative inoculum which had an established capability to naturally infect a ruminant species and to form AE lesions. A bovine-derived strain was used at that time in the absence of a characterised *E. coli* strain known to form AE lesions in sheep. In Lamb 4 the inoculum was pre-incubated in DMEM, to promote expression of LEE genes (Kenny et al. 1997a). This, it was hypothesised, would increase the number of bacteria adhering to the mucosa within the incubation period.

4.2 Materials and Methods

Animal procedures

For ethical and practical reasons, continuous anaesthesia was employed, and a six-hour incubation period was chosen to give the inoculated organisms adequate time to form AE lesions, based upon the confirmatory fluorescence actin staining (FAS) performed with these strains *in vitro*. (Section 3.5.2C). The colon was selected for the creation of the loops because *E. coli* O157:H7 has formed lesions in the colon of calves (Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1999) and neonatal lambs (Chapter 5). The spiral colon specifically was used in view of its suitability in respect of surgical access and anatomical features, i.e. it is readily exteriorised and manipulated, is of relatively narrow calibre, and provides the maximum scope for multiple loops within the large intestine by virtue of its length.

The technique employed is described in detail in Section 2.6.3.2, and a schematic illustration of the loops formed is shown in Figure 4-a. Four lambs, aged between five and six months, were used on separate occasions. Lamb 1 was inoculated with PBS only. Inocula for the other lambs are shown in Table 4-A. Details of the sources of the strains used are in Section 2.2.1. Genotypic characteristics of the Shiga toxin-producing *E. coli* O157:H7 and the *E. coli* O80 strains are given in Table 3-B. Strain EC1537 possesses genes encoding Stx1 (Gunning et al. 2001), the LEE, enterohaemolysin and a serine protease (*katP*) (Pearson et al. 1999). Strain NCTC 12900 has a typical *E. coli* O157:H7 genotype in respect of LEE and virulence plasmid genes, but lacks Shiga toxin (Dibb-Fuller et al. 2001).

Table 4-A: Details of inocula for ligated colon loops

Lamb	Inoculum type	Strains used	Serotypes
2	non Shiga toxin-producing AEEC in PBS carrier	NCTC 12900	O157:H7
		85440	O80:NM
		B171	O111:NM
3 and 4	AEEC in PBS (Lamb 3) or DMEM (Lamb 4) carrier. Strains for Lamb 4 pre-incubated in DMEM	EC1537	O26:H11
		EC218	O157:H7
		EC222	O157:H7
		140065	O157:H7
		EC157	O157:H7
		NCTC 12900	O157:H7

Pathological procedures

Tissues were fixed, processed and examined as described in sections 2.7.1, 2.7.2, 2.7.3, and 2.7.4.2A respectively.

Bacteriological procedures

Bacteriological procedures are described in sections 2.2.9 (screening faeces for *E. coli* O157:H), 2.2.6 (preparation of inocula) and 2.2.8 (TVC determination). The wild-type unmarked *E. coli* O157:H7 strains were used in lambs 3 and 4 in preference to their antibiotic resistance marked derivatives. This was because there was no need for recovery and differentiation between strains in the protocol used, and therefore any phenotypic alterations associated with antibiotic resistance marking could be avoided.

4.3 Results

Animal procedures

All four lambs survived the anaesthetic and surgical procedures until euthanasia following excision of the colon loops. It proved possible to create twelve loops of approximately 10 cm length in the spiral colon, providing six test loops inoculated with bacteria plus six control loops inoculated with carrier medium only, each control loop being immediately proximal to its respective test loop. Only 10 loops were created in Lamb 1. The inoculation procedure was associated with minimal leakage of the inoculum from the

injection site, and thermocautery assisted in limiting this. The colon loops were opened and placed in fixative within two minutes of excision.

Bacteriological findings

E. coli O157 was not detected in faecal samples taken from any of the test animals on any of the three occasions over a four month period prior to the experiments.

For lambs 3 and 4, inoculated with STEC, TVC were performed on the inoculum preparations, and these are listed in Table 4-B.

Macroscopic pathological findings

Upon removal from the animal, the serosa of the ligated loops appeared reddened and was often mildly oedematous. The luminal contents were mucoid and frequently were bloodstained. Loops did not appear dilated and no differences between control and test loops were seen.

Histopathological findings

Lamb 1:

The loops, all inoculated with PBS only, were numbered from 1 to 10 in sequential progression from proximal to distal spiral colon. Five loops (1, 3, 5, 7 and 9) were examined. The epithelium was uniformly well preserved, with no evidence of detachment or degeneration. The lamina propria appeared mildly oedematous in the more distal loops. Small, focal haemorrhages and more extensive areas of haemorrhage were common in the lamina propria. In addition, locally extensive haemorrhages were observed on the serosa, there was a small amount of free blood in the lumen and haemorrhages were occasionally seen in mucosa-associated lymphoid tissue. Mild or moderate apoptosis was common in lymphoid follicles. In the serosa and muscularis externa, acute inflammatory changes, comprising moderate neutrophil margination, extravasation and migration, were widespread.

Lamb 2:

Details are presented in Table 4-C. Tissue preservation was uniformly excellent, and haemorrhages were rarely seen. Inflammatory and apoptotic changes similar to Lamb 1 were seen in the outer layers of the colon wall and in the mucosal lymphoid tissue respectively. The bacterial populations at the mucosal surface varied considerably, in both inoculated and control sections. Some loosely-attached bacteria were seen in the NCTC 12900-inoculated loop. No AE-type lesions were seen in any section.

Lambs 3 and 4:

Details are presented in tables 4-D and 4-E. Since the same strains were used in both animals, these two animals are considered together and the twelve bacteria-inoculated loops have been lettered consecutively (Table 4-B) for reference. The order of inoculation of loops, from the cranial to the caudal end of the spiral colon, follows alphabetical sequence in each animal, i.e. each strain was inoculated into the same relative position in the colon in each animal. As these lambs were inoculated with *E. coli* O157:H7, more extensive scrutiny of the tissues was performed than with the previous two lambs. Fifteen to 21 sections per loop were examined, and these were prepared from between six and 11 pieces of embedded tissue, with several sections taken from each piece using different levels in the wax block. When measured macroscopically, the microscope sections ranged in length from 11 to 59 mm. Preservation of the mucosa was generally good, with only one loop (L) exhibiting detachment of short sections of the epithelium. AE-type lesions were found in H&E-stained sections in several loops inoculated with bacteria. Similar lesions were not observed in control loops. The occurrence of lesions is summarised in Table 4-B. Lesions were extremely sparse and typically were small, covering one or two adjacent enterocytes, with individually distinguishable bacteria (Figure 4-b). Some lesions were found on detaching enterocytes (Figure 4-c), although only in Loop D was this exclusively the case. The mean frequency of occurrence of AE lesions per section was 0.21 (82 lesions / 389 sections). However, a tendency for clustering of lesions was observed in some loops, with up to seven lesions being observed on a single section (Table 4-B), which is 33 times greater than the mean. Clusters of lesions were not associated with areas of visibly damaged epithelium.

The epithelial morphology appeared abnormal in four out of 12 loops in Lamb 3, two of which were controls not inoculated with bacteria, and in 11 out of 12 loops in Lamb 4. The mucosal surface in affected loops had a ragged profile in a multifocal pattern. In Lamb 4, this was often accompanied by rounding up and detachment of enterocytes, both singly and in small clusters. Additionally, in Lamb 4, small foci of epithelial necrosis were present in two control loops. A number of other histological features, including focal mucosal haemorrhage, neutrophilic serositis, and apoptosis in mucosa-associated lymphoid tissue, were commonly seen in both inoculated and control loops, similarly to lambs 1 and 2. In loops L, K and J in Lamb 4, localised colonisation of the (subepithelial) lamina propria by bacteria was evident near some areas of epithelial erosion. In Loop E a modest increase in the number of neutrophils in the lamina propria and several foci of neutrophils within the luminal epithelium were seen. In Lamb 4 both inoculated and control loops contained slender organisms of spiral morphology, in moderate or large numbers, occupying crypts and the intestinal lumen. Loose aggregations of such organisms were frequently present on the mucosal surface (Figure 4-e), but there was no evidence that these organisms were attached

to or invading the host intestinal wall, and no specific association with areas of epithelial abnormality was detected. These bacteria stained positively with a Warthin-Starry silver stain.

Immunohistochemical findings

The ipx technique proved sensitive and specific using the primary antisera for *E. coli* O157 and O26 at dilutions of 1:1000 and 1:4000 respectively. The O80 antiserum was not tested with a positive control preparation and was used at a 1:500 dilution, this having not yielded any non-specific staining of O157 organisms in a control section. Details for individual sections are included in tables 4-C, 4-D and 4-E.

Lamb 2:

Immunostaining for O80 and O157 antigens was performed on tissue from two loops, wherein bacteria loosely-attached to the mucosa had been seen in H&E-stained sections. Positive staining was not observed.

Lambs 3 and 4:

Ten to 20 sections per loop were immunostained, and the results are presented in Table 4-B. AE-type lesions were found in five of the six loops in which lesions had been detected in H&E-stained sections. In addition, AE lesions were found in four loops in which such lesions had not been detected in H&E-stained sections. Positive staining confirmed that the lesional bacteria were *E. coli* O157 (Figure 4-d) or O26 (Figure 4-e), as appropriate to the inoculum. A few non-staining closely adherent bacteria were seen in association with O26 lesions. In a number of loops, single positively-staining bacteria were seen lying on the mucosal surface. As the nature of the attachment, if any, of these single organisms to the host cells was uncertain, the recording of an AE lesion required the identification of a cluster of two or more positively-staining bacteria in close apposition to the mucosa, i.e. evidence of multiple attached organisms in a single focus. Occasionally, positively-staining attached bacteria were seen where, upon initial examination of a corresponding H&E-stained section, only surface debris had been observed (Figure 4-g).

Substantial numbers of bacteria expressing O157 antigen (Figure 4-f) were detected in the subepithelial lamina propria of the three loops (J, K, L) in which lamina proprial colonisation had been detected using H&E-stained sections, and additionally in Loop I. The concentration of O157 organisms in the lamina propria was high (i.e. dense, uncountable masses of bacteria) when compared with that in the associated luminal contents.

Table 4-B: Details of attaching-effacing (AE) lesions in histological sections of ligated spiral colon loops of two lambs inoculated with *E. coli* O157:H7 and O26:H11

Lamb	Serotype	Strain	Loop	Inoculum dose ($x10^8$ cfu)	No. of AE lesions (no. of sections examined)		Max. lesions per section	Total sections with AE lesions	**Total AE lesions**
					H&E*	ipx**			
3	O26:H11	EC1537	A	5.0	8 (18)	11 (13)	7	10	**19**
	O157:H7	EC218	B	8.3	6 (21)	20 (14)	7	13	**26**
		EC222	C	4.5	0 (18)	0 (12)	0	0	**0**
		140065	D	4.6	1 (21)	0 (10)	1	1	**1**
		EC157	E	6.3	0 (16)	3 (12)	2	2	**3**
		NCTC 12900	F	5.7	0 (22)	0 (12)	0	0	**0**
4	O26:H11	EC1537	G	12.3	0 (16)	2 (14)	1	2	**2**
	O157:H7	EC218	H	12.7	4 (18)	5 (18)	2	8	**9**
		EC222	I	14.7	0 (15)	5 (14)	5	1	**5**
		140065	J	10.6	3 (21)	4 (20)	1	7	**7**
		EC157	K	10.3	0 (16)	2 (14)	2	1	**2**
		NCTC 12900	L	9.3	3 (18)	5 (16)	2	5	**8**
				Totals:	**25 (220)**	**57 (169)**		**50**	**82**

* H&E: haematoxylin and eosin-stained sections. **ipx: immunoperoxidase-stained sections.

Electron microscopic findings

Nine pieces of tissue (detailed in tables 4-D and 4-E) were processed using the wax block retrieval technique from lambs 3 and 4, seven from loops demonstrating light microscopic evidence of AE lesions. Three of these seven specimens yielded sections containing bacteria which had formed AE lesions. These were: two from Loop A, containing *E. coli* O26 (Figure 4-h), and one from Loop H, containing *E. coli* O157:H7 (Figure 4-i). From one of the two resin blocks prepared from areas where lamina proprial colonisation by bacteria had been seen, organisms with a morphology consistent with *E. coli* were seen around and within the basal portions of crypt cells and in areas of haemorrhage. In addition, curved or spiral bacteria were seen (Figure 4-j). In sections from the other block, bacteria were seen within a goblet cell secretory vacuole and a neutrophil.

4.4 Discussion

E. coli O157:H7 formed AE lesions within six hours in ligated spiral colon loops of weaned lambs and, where examined, the light microscopic appearance of AE lesions was confirmed by TEM of tissue retrieved from wax blocks. Electron microscopy proved demanding in the face of small and sparse lesions and only three of seven attempts to extract lesions for examination were successful. Lesions were not seen with strains B171 (*E. coli* O111:NM, human EPEC) and 85440 (O80:NM, bovine EPEC), but relatively few sections of these loops were examined, in comparison with the *E. coli* O157:H7-inoculated loops. The blood supply to the loops appeared to be sufficient to preserve the mucosa adequately for six hours, but changes that may have been caused by circulatory compromise include the morphological alterations to the mucosa, observed in both control and test loops, and the limited mucosal haemorrhage.

There was no evidence of a loop-dilating effect of any inoculum, nor of morphologic alterations specifically associated with any STEC inoculum. The effect of Shiga toxin upon the bowel has been shown to vary between species (Tzipori et al. 1987; Richardson et al. 1992), and this includes changes observed in ligated loops (Williams Smith et al. 1983; Mobassaleh et al. 1988). It is consistent with the observed lack of clinical disease in naturally (Kudva et al. 1996; Kudva et al. 1997a) and experimentally (Kudva et al. 1995; Kudva et al. 1997b; Cornick et al. 2000; Wales et al. 2001) STEC-colonised sheep that the ovine colon appeared to be resistant to Shiga toxin-induced changes for the six-hour exposure used in the present studies. However, the elaboration of Shiga toxins was not assessed. Current models of the formation of AE lesions do not propose a role for Shiga toxin (Frankel et al. 1998b) and there is no evidence from the present study that Shiga toxins are directly involved in the formation of AE lesions. This does not however preclude a role for the toxin in the

colonisation of animals, and indeed other workers (Dean-Nystrom et al. 1999) have suggested that they might facilitate colonisation.

The sensitivity of ipx staining for the detection of AE lesions appeared to be superior to H&E, but this was not amenable to statistical analysis with the present data, given the variability in sizes of the sections examined. Numerous sections potentially containing small and sparse lesions were being screened, and it proved easier to detect specifically-stained bacteria at the mucosal surface in ipx-stained sections than to detect non-specifically stained intimately-adhering bacterial clusters in H&E-stained sections. Therefore, it is not surprising that the number of AE lesions detected per H&E-stained section (0.11) was substantially lower than the corresponding number for immunostained sections of a similar size (0.34).

Different patterns of lesion formation were seen between strains and between inoculation regimes in lambs 3 and 4. However, the low incidence of lesions in this instance means that a great many more sections would need to be examined to provide unequivocal evidence of differences. Comparisons between findings in the two lambs are aided by the fact that the strains were placed in the same locations relative both to each other and to the colon in the two lambs. For the O157:H7 strains in lambs 3 and 4, a similar number of lesions were recorded, but in the former lamb these were predominantly formed by one strain (EC218), with few or no lesions by the others, whereas in the latter the lesions were more evenly distributed between strains. In addition, there was a marked difference in the number of *E. coli* O26:H11 lesions between the two lambs whereby, in contrast to the *E. coli* O157:H7-inoculated loops, the PBS inoculation regime yielded more O26-associated lesions. Overall, there is no evidence that the *E. coli* O26:H11 bovine pathogen formed lesions more readily than did the *E. coli* O157:H7 strains, nor that there was a consistent difference between the two serotypes in response to DMEM induction. Indeed there appears to be as much variation amongst *E. coli* O157:H7 strains as between the two serotypes. No consistent effect of variation in the inoculum dose (albeit over only a fourfold range) was seen.

The potential distorting effect of the clusters of lesions seen in some loops upon the total numbers of lesions detected is difficult to quantify with the modest numbers involved, but the clustering effect limits useful comparisons between loops. The clusters were not associated with any visible predisposing features, such as mucosal damage, and may have formed consequent upon localised high concentrations of inoculated organisms.

The identity of the numerous slender, spiral organisms seen in crypts and at the mucosal surface in Lamb 2 is unknown, but *Campylobacter*-like organisms have been seen on the mucosa of sheep naturally-infected with *Campylobacter fetus* (Jopp and Orr 1980). Although there was no evidence for associated pathological change, these spiral organisms remain a potential confounding factor when comparing data from the two lambs, and further studies comparing inoculation regimes within the same animal would be required to address this.

The present data is insufficient to speculate about a possible role of these organisms in colonisation by *E. coli* O157:H7 and other AE bacteria.

The bacterial colonisation of the lamina propria, with electron microscopic evidence of a host response of phagocytosis, was only seen in one experimental animal (Lamb 4) and it did not correlate specifically with the presence of AE lesions. It was associated with the inoculated *E. coli* O157:H7 organisms, of both Shiga toxin-producing and non Shiga toxin-producing strains but was not observed in *E. coli* O26-inoculated loops. The high concentration of O157 organisms in the lamina propria suggests that the O157 bacteria colonised the tissue more avidly than did other bacteria present in the lumen, although spiral bacteria were also seen in the tissue in EM preparations. Pre-incubation in DMEM may have promoted the expression of bacterial factors in Lamb 4 that were necessary for colonisation. In addition, the occurrence of observed epithelial abnormalities was highest in this lamb and, furthermore, this was the only lamb in which the *Campylobacter*-like organisms were seen. However, the significance of these two observations in relation to the lamina proprial colonisation is undetermined. Colonisation of the lamina propria in animals inoculated experimentally with STEC O157:H7 has been reported in the caeca of chicks at 14 and 28 days p.i. (Beery et al. 1985) and of gnotobiotic piglets at 5 days p.i. (Tzipori et al. 1986).

The immune status of the animals was not investigated in this study. Other workers (Li et al. 2000) have demonstrated that strong serological responses to proteins involved in the formation of the AE lesion occur in humans clinically affected with *E. coli* O157:H7 and it follows, therefore, that the immune status of the sheep may have played a role in limiting intimate adherence. Although the sheep were young and were demonstrated to be free from *E. coli* O157:H7, it remains possible that previous exposure to heterologous AEEC (such as those reported Experiment 6/3, Chapter 6) may have induced cross-protective immunity.

Conclusion

The present ligated loop studies have demonstrated the potential susceptibility of weaned lambs to the attachment and formation of AE lesions in the large intestine by *E. coli* O157:H7. There is no convincing evidence that, in this experimental system, the bovine pathogen *E. coli* O26:H11, strain EC1537, formed AE lesions more readily than did *E. coli* O157:H7. It was shown that extensive, detailed examination of tissues was required for the reliable detection of specific bacterial attachment, and there was an indication of the relative sensitivities of H&E and ipx stains for the detection of small, sparse AE lesions.

Table 4-C: Histopathological findings from Lamb 2

Serotype	**Controls (uninoculated)**	**O157:H7**	**O80:NM**	**O111:NM**
Strain		*NCTC 12900*	*85440*	*B 171*
Loops numbered	1, 3, 5, 7, 9, 11	2, 8	4, 10	6, 12
Number of sections	2 each (H&E) 6 ipx for Loop 3	5 +4 (H&E) 5 + 4 (Giemsa) 10 + 18 (Ipx)	6 + 4 (H&E)	2 + 2 (H&E)
Preservation	Excellent.	Excellent.	Excellent.	Excellent.
Haemorrhage	In lamina propria and on serosa of Loop 5	None.	None.	None.
Epithelial morphology	NAD	NAD	NAD	NAD
Histological features	Moderate PMN infiltration of serosa, subserosal tissue and muscularis externa. Moderate apoptosis in lymphoid nodules.	As controls. Scattered PMN in lamina propria of Loop 2.	As controls.	As controls. Also localised submucosal oedema.
Bacterial features	Loop 3 has many rod-shaped bacteria at the luminal surface - possible mix-up of loops 3 and 4 samples, ipx with O80 antiserum is negative.	Loop 2: patchy, loose mat of bacteria over epithelium, some appearing loosely attached. Loose bacterial clusters in some crypts. No specific staining by O157 ipx. Loop 8: occasional single bacteria and small clusters in crypts.	No bacteria seen.	A few luminal rod bacteria in Loop 6. No evidence of attachment.

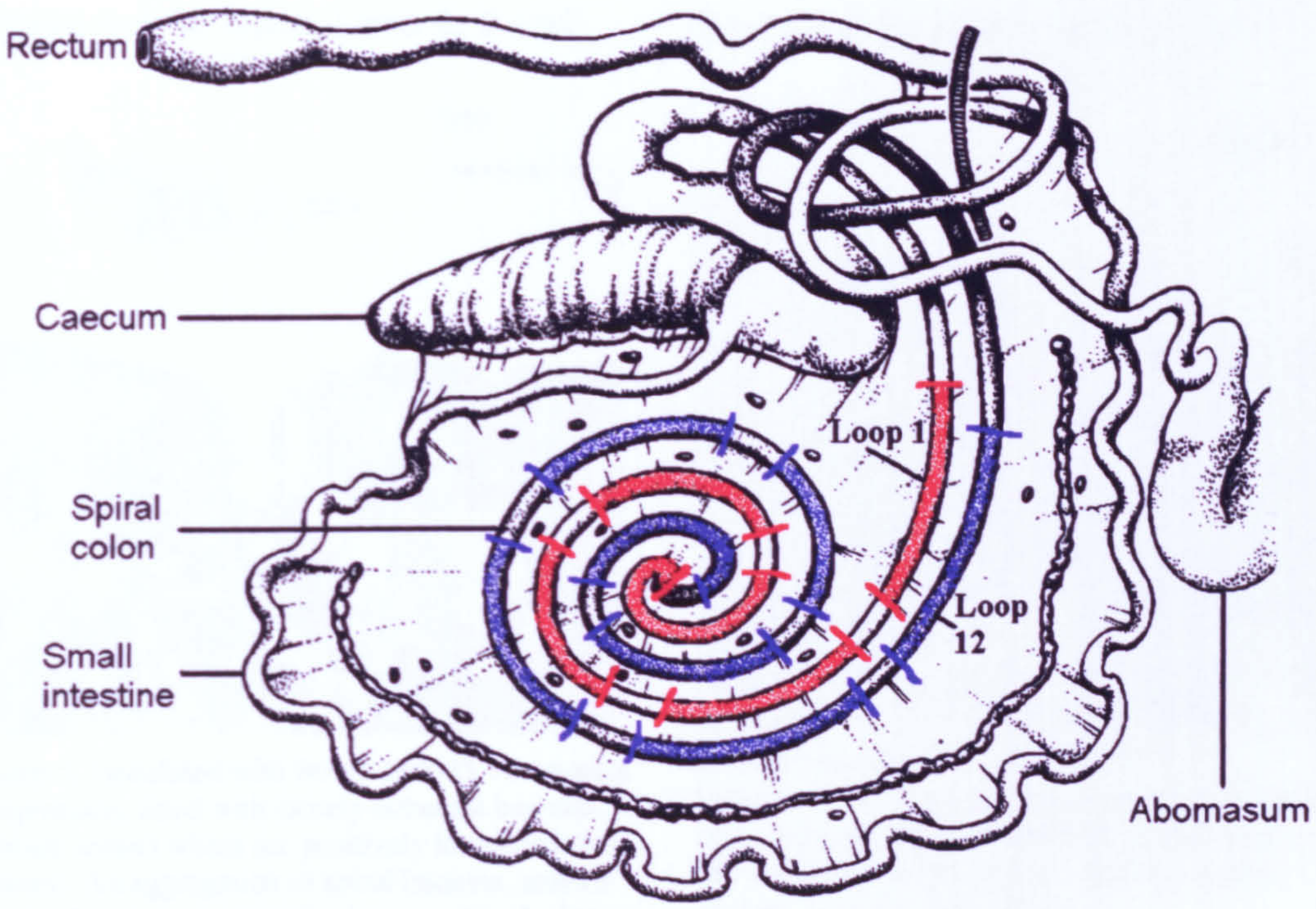
Table 4-D: Histopathological findings from Lamb 3

Serotype	**Controls**	**O26:H11**	**O157:H7**				
Strain		*EC1537*	*EC218*	*EC222*	*140065*	*EC157*	*NCTC 12900*
Loops numbered	1, 3, 5, 7, 9, 11	2	4	6	8	10	12
Blocks and number of sections	6 x A (6 loops)	A,B,C,D	A, B, C	A, B, C, D, E	A, B, C	A, B, C, D, E	A, B, C, D
	12 H&E	18 H&E	21 H&E	18 H&E	21 H&E	16 H&E	22 H&E
		13 Ipx	14 Ipx	12 Ipx	10 Ipx	12 Ipx	12 Ipx
Preservation	Good	Good	Good	Good	Good	Good	Good
Haemorrhage	1 focus in LP in 1/6 loops	1 mucosal focus	None seen	None seen	1 focus in LP	None seen	1 focus in LP
Epithelial morphology	Locally ragged in 2/6 loops	Normal	Locally ragged	Locally ragged. Artefactual loss in 1 section.	Normal	Normal, artefactual loss in 1 section.	Normal
Histological features	PMN serositis in 6/6 loops, scattered to moderate apoptosis in LP lymphoid aggregates.	PMN serositis, mucus & haemorrhage in lumen.	PMN serositis	PMN serositis, apoptosis in mucosal lymphoid aggregates.	PMN serositis.	PMN serositis. PMN's increased in LP and focally transmigrating across epithelium.	PMN serositis
Bacterial features	NAD	AE-type lesions: 7 H&E, 11 ipx O26 positive sites. Typically well-separated bacteria, covering 1-2 host cells.	AE-type lesions, 6 H&E, 21 ipx O157 positive lesions, plus 10 small clusters of attached ipx +ve cells, possibly AE lesions.	No attached bacteria seen. Ipx O157 positive cells present adjacent to epithelium in lumen.	One AE-type lesion, H&E. 2 ipx O157 positive cells attached to surface in mucosal cleft (possible AE).	AE-type lesions, 3 O157 ipx positive. Also ipx positive cells in surface mucus.	Scattered bacteria in crypts. 1 O157 cell attached to surface, 3 in a crypt, but no clear AE type lesions.
Electron microscopy	No	2 blocks, both show AE lesions	No	No	No	No	No

Table 4-E: Histopathological findings from Lamb 4

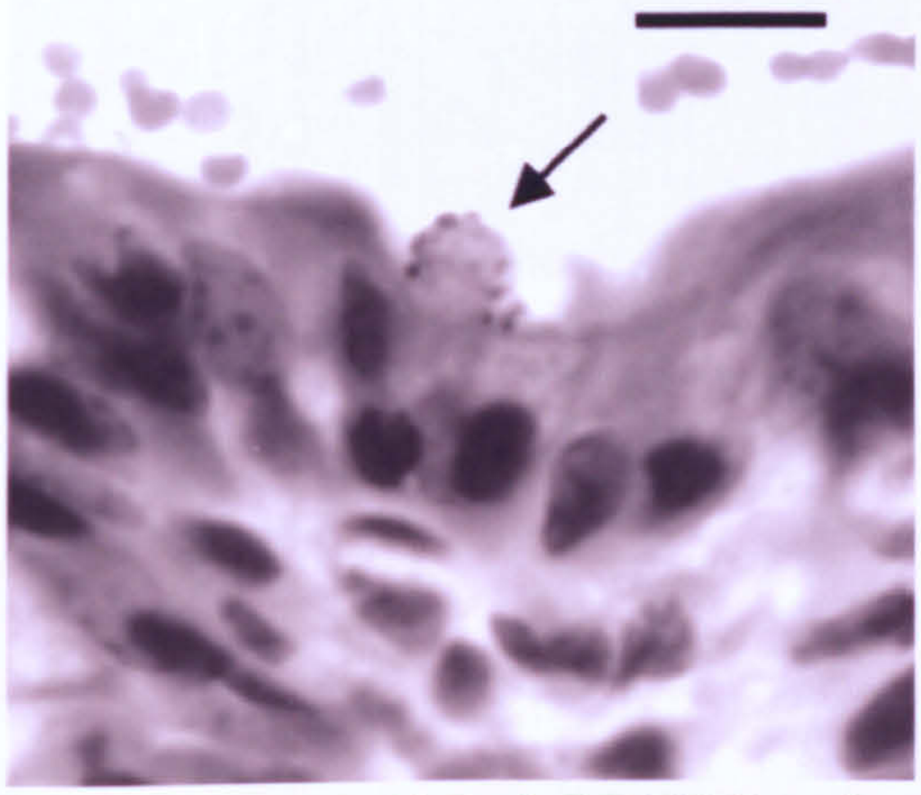
Serotype	**Controls**	**O26:H11**	**O157:H7**				
Strain		*EC1537*	*EC218*	*EC222*	*140065*	*EC157*	*NCTC 12900*
Loops numbered	1, 3, 5, 7, 9, 11	2	4	6	8	10	12
Blocks and number of sections	6 x A (6 loops)	A, B, C	A, B, C, D	A, B, C	A, B, C, D	A, B, C	A, B, C
	12 H&E	16 H&E	18 H&E	15 H&E	21 H&E	16 H&E	18 H&E
		14 Ipx	18 Ipx	14 Ipx	20 Ipx	14 Ipx	16 Ipx
Preservation	Good	Good	Good	Good	Good	Moderate	Moderate
Haemorrhage	None seen	In lumen	None seen	None seen	None seen	None seen	None seen
Epithelial morphology	Scattered foci of epithelial necrosis and detachment 2/6. Focally ragged, some detaching cells. 3/6	Focally ragged, rounding up and detaching in places.	Frequent single or small clusters of detaching cells, NOT autolytic appearance.	Multifocal areas of raggedness and erosion	Ragged, detachment of single cells.	Ragged. Frequent detachment of single cells and small clusters.	Ragged, single cells detaching and focal detachment of short strips.
Histological features	PMN serositis with perivascular migration. Scattered LP necrosis 3/6	PMN serositis, apoptosis in lymphoid aggregates	PMN serositis, apoptosis in lymphoid aggregates.	PMN serositis, apoptosis in lymphoid aggregates. Abundant mucus	PMN serositis. Patchy, mild infiltration of LP by PMN.	PMN serositis.	PMN serositis.
Bacterial features	Many bacteria in lumen and in crypts, also loosely attached to mucosa.	AE-type lesions: two O26-positive ipx. Many bacteria in lumen and crypts.	AE-type lesions: four H&E, four O157-positive ipx.	AE-type lesions: five O157-positive ipx, same lesions under debris on H&E. Many bacteria at luminal surface and in crypts. Ipx O157 positive bacteria invading LP and attached to detached epithelial cells.	AE-type lesions three H&E, four O157-positive ipx. O157 ipx-positive bacteria colonising upper LP. Many bacteria at luminal surface and in crypts.	AE type lesions: two O157-positive ipx. Patchy colonisation of upper LP by O157 +ve bacteria	AE type lesions: three H&E, five O157-positive ipx. Many bacteria in crypts. Localised colonisation of LP by ipx O157 positive bacteria.
Electron microscopy	No	No	2 blocks, 1 shows AE lesions	1 block	3 blocks	1 block, shows bacteria in LP	No

Figure 4-a: Schematic view of intestinal loop locations



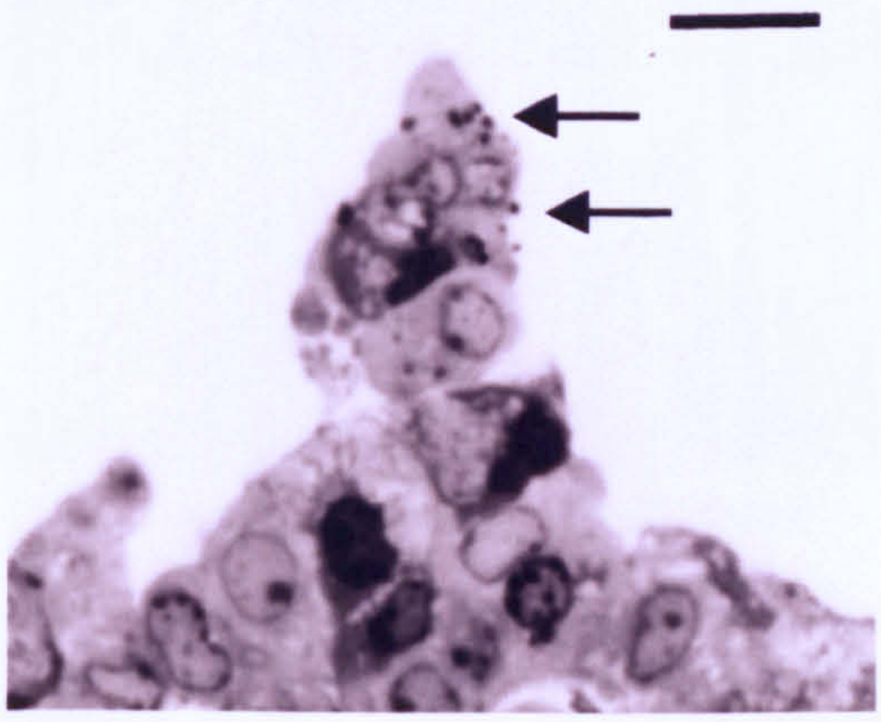
Ligated intestinal loops were created in both the centripetal (red loops) and centrifugal (blue loops) parts of the spiral colon.
Figure adapted from Dyce et al. (1987).

Figure 4-b: AE lesion, Lamb 3, *E. coli* O26:H11



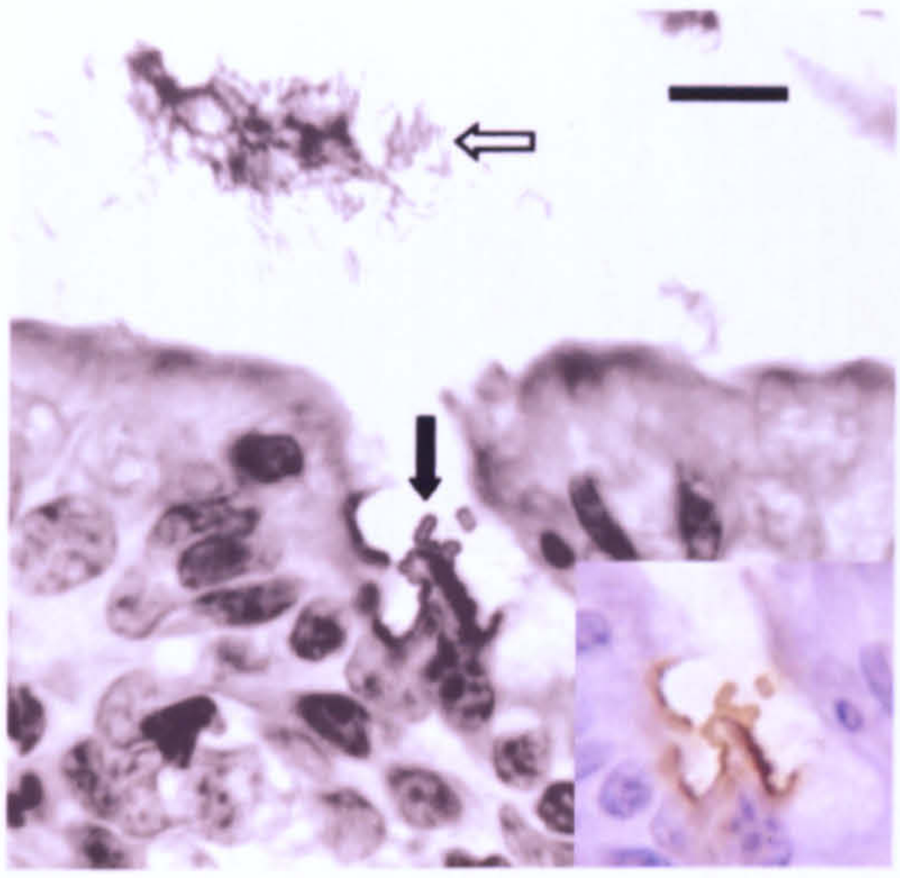
Loop A, inoculated with Strain EC1537. Bacteria are closely adherent to the apical membrane of an enterocyte (arrow), which protrudes above the intestinal surface. H&E, bar = 8 μm.

Figure 4-c: AE lesion, Lamb 4, *E. coli* O157:H7



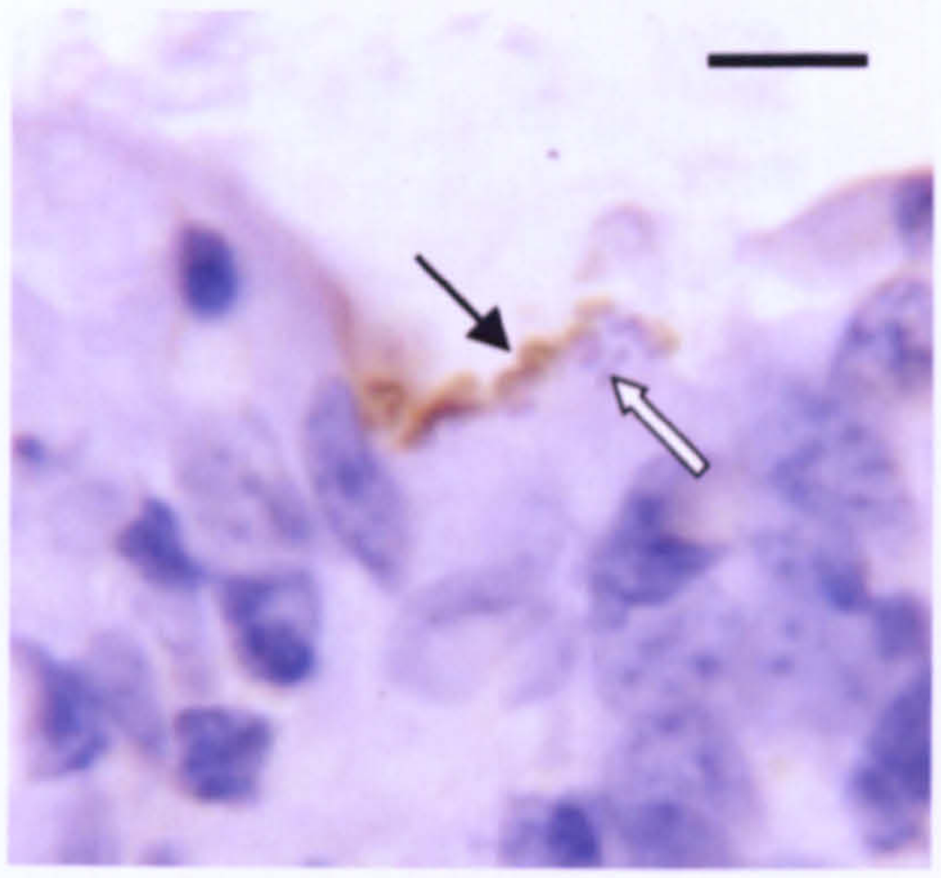
Loop H, inoculated with Strain EC218. Degenerating and detaching enterocytes are colonised by bacteria (arrows). Toluidine blue stained resin-embedded section, bar = 10 μm.

Figure 4-d: AE lesion, Lamb 4, *E. coli* O157:H7



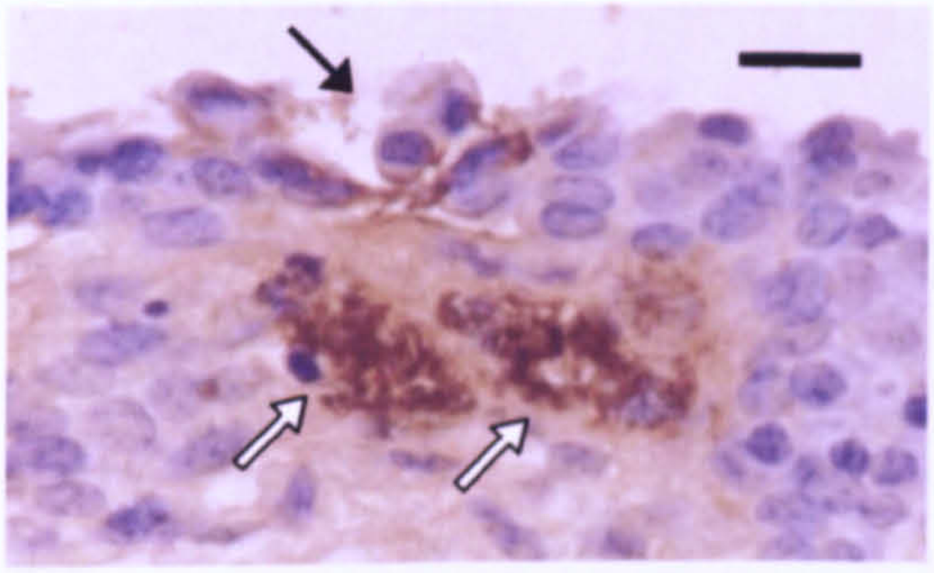
Loop J, inoculated with Strain 140065. A mucosal depression, lined with closely-adherent bacteria (black arrow) which are positively immunostained (inset). An aggregation of spiral bacteria, stained with Mayer's haematoxylin, is present in the lumen (white arrow). O157 ipx, bar = 8 μm.

Figure 4-e: AE lesion, Lamb 3, *E. coli* O26:H11



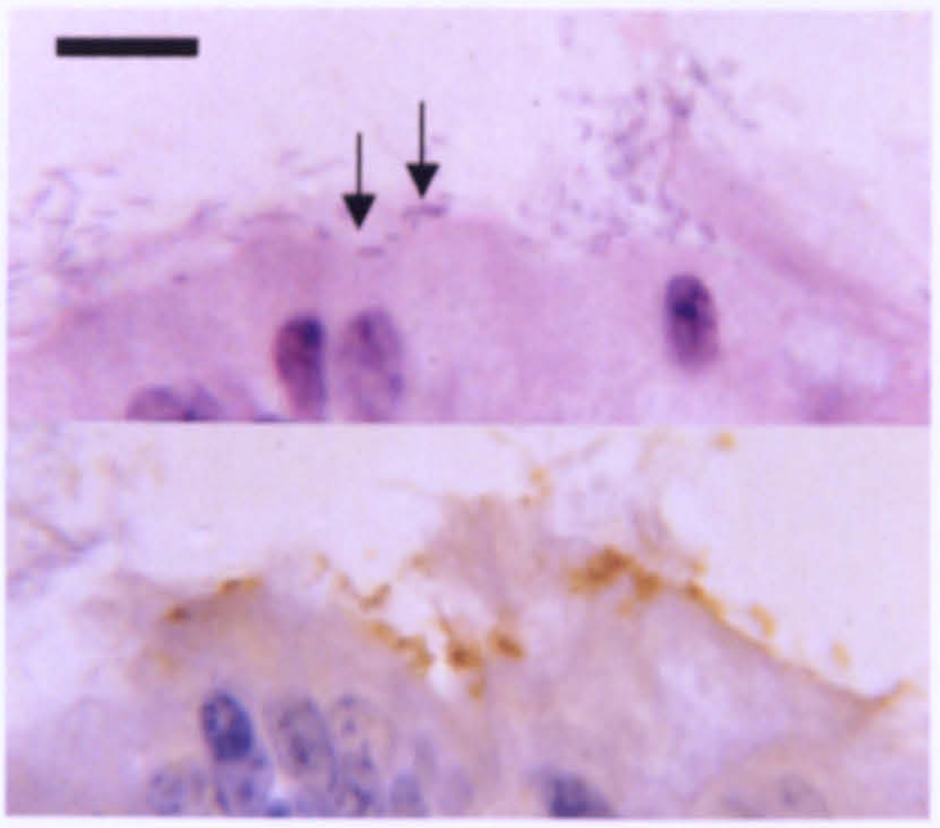
Loop A, inoculated with Strain EC1537. Immunostained bacteria (black arrow) are closely adherent to the apical membrane of enterocytes. A few unstained bacteria (white arrow) are also present. O26 ipx, bar = 8 μm.

Figure 4-f: *E. coli* O157:H7 in lamina propria of Lamb 4



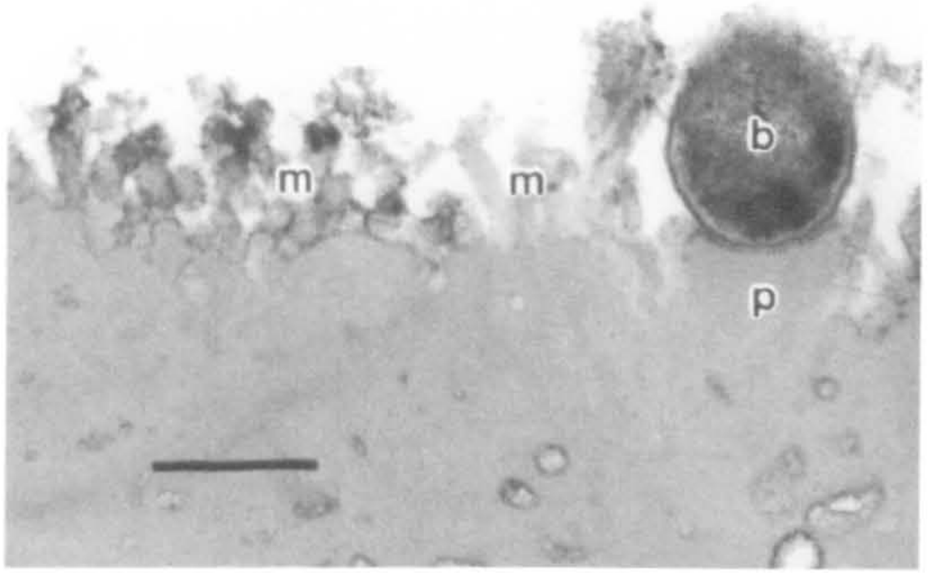
Loop L, inoculated with Strain NCTC 12900. Positively-stained bacterial masses (white arrows) are present in the lamina propria, underlying an area of epithelial disruption (black arrow). O157 ipx, bar = 13 μm.

Figure 4-g: H&E- and ipx-stains, Lamb 4, *E. coli* O157:H7



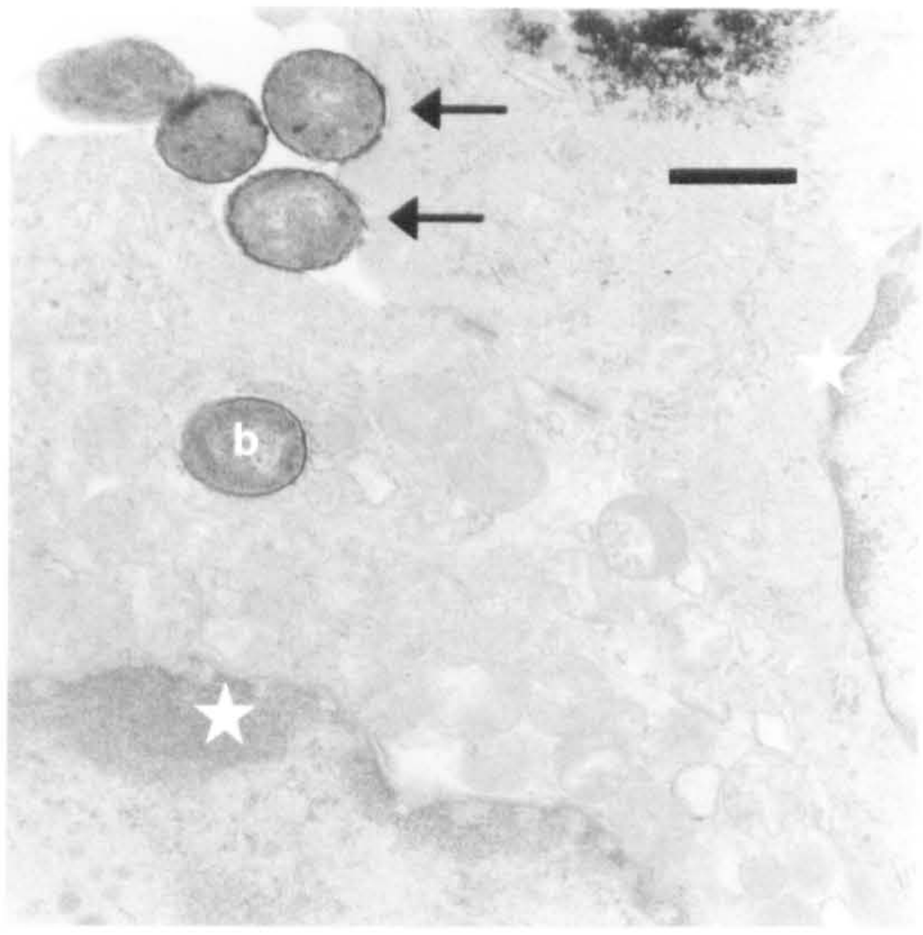
Loop I, inoculated with Strain EC222. Upper (H&E) and lower (ipx) pictures are of close (almost consecutive) sections from the same tissue block, and are from corresponding locations in the respective sections. Possibly adherent bacteria are present in the H&E-stained section (arrows), but only the ipx stain highlights the location and identity of O157 organisms (brown) amongst the debris at the mucosal surface. H&E / ipx, bar = 10 μm.

Figure 4-h: AE lesion, Lamb 3, *E. coli* O26:H11



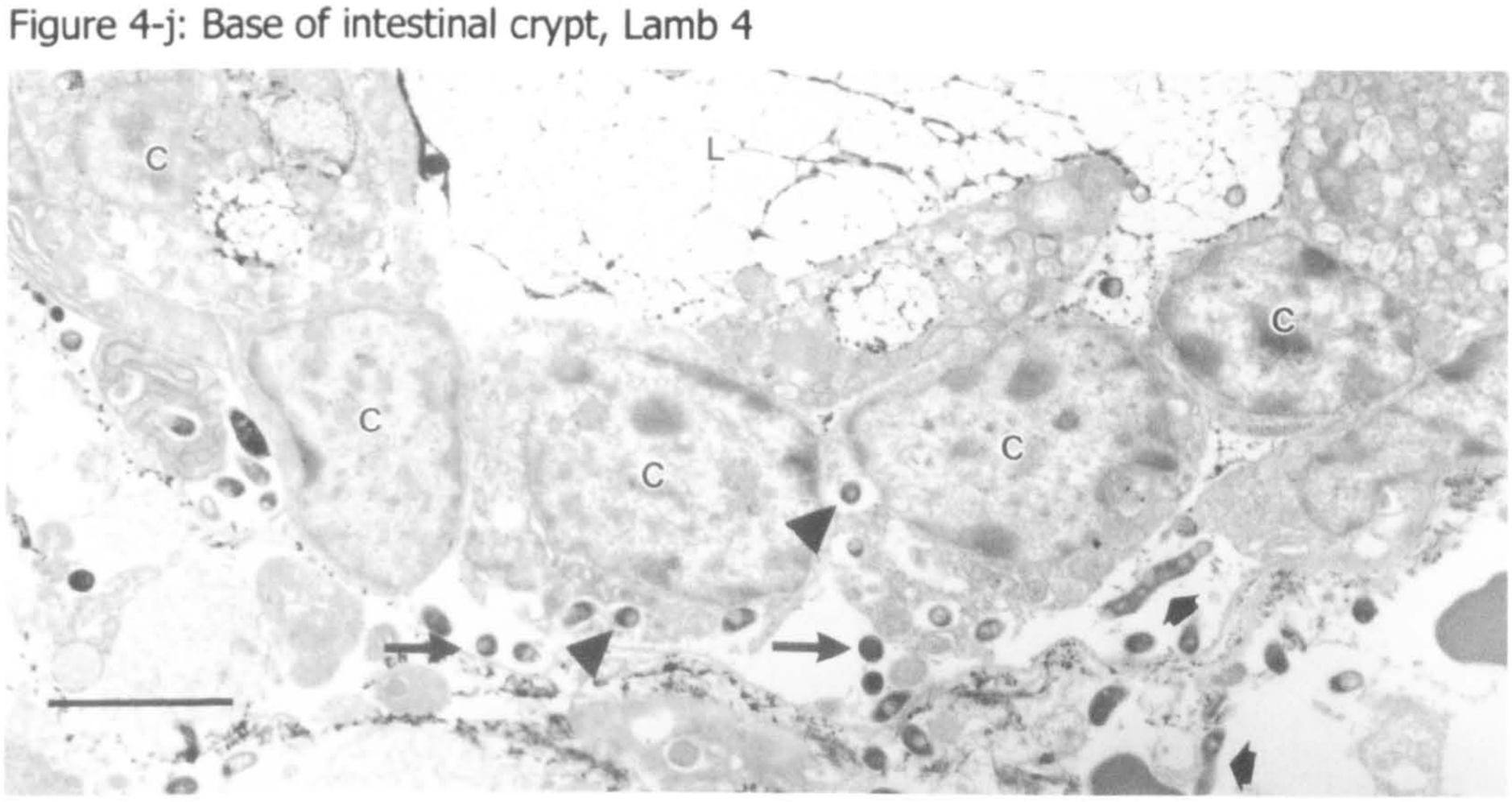
Loop A, inoculated with Strain EC1537. Microvilli (m) are effaced around a pedestal (p) to which a bacterium (b) is intimately attached. Bar = 500 nm.

Figure 4-i: AE lesion, Lamb 4, *E. coli* O157:H7



Loop H, inoculated with Strain EC218. Same lesion as Figure 4-c. AE bacteria (arrows) are present on two adjacent, degenerating enterocytes (asterisks). There is also possible invasion of one of the enterocytes by a bacterium (b).
Bar = 750 nm.

Figure 4-j: Base of intestinal crypt, Lamb 4



Loop K, inoculated with inoculated with *E. coli* O157:H7 Strain EC157. Bacteria of *E. coli* morphology are present beneath (long arrows) and apparently within (arrowheads) crypt epithelial cells (C). Spiral bacteria (short arrows) are also present in the lamina propria. L: crypt lumen. Bar = 4 μm.

Chapter 5 - NEONATAL LAMB STUDIES

5.1 Introduction

The contribution of mucosal adherence to the persistence of *E. coli* O157:H7 in ruminants is the primary focus of the present studies. *E. coli* O157:H7 possesses the well-defined AE capability (Sherman et al. 1987) plus other potential adhesins including: outer membranes (Sherman and Soni 1988b), lipopolysaccharide (Paton et al. 1998), exopolysaccharide (Junkins and Doyle 1992) and the IrgA homologue adhesin (Tarr et al. 2000). There are also 14 putative fimbrial operons identified on the genome of an *E. coli* O157:H7 outbreak strain (Hayashi et al. 2001). Adhesion of *E. coli* O157:H7 to the recto-anal mucosa of a persistently colonised steer has recently been demonstrated (Naylor et al. 2003). In cattle the principle of intestinal susceptibility to *E. coli* O157:H7 AE lesions has been shown experimentally using a high inoculum dose of 10^{10} cfu in young animals, either neonates (Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1998) or fasted three- to four-month old calves (Dean-Nystrom et al. 1999). It may be that such lesions do occur in persistently-colonised animals, but that detection is currently only possible when young animals, likely to be more susceptible to lesion formation due to the immunological and/or physiological immaturity of the alimentary tract, and a high-dose inoculum are used. Studies examining potential *E. coli* O157:H7 adhesion in the intestine of neonatal lambs have not been reported. Reports of experimental investigations into *E. coli* O157:H7 interactions with sheep are summarised in Section 1.4.2.7.

In initial studies using six-month and six- to 11-week old lambs inoculated with *E. coli* O157:H7 (experiments 6/1 and 6/2, Chapter 6), evidence of attachment of the experimental strains to the intestinal mucosa was not found. Therefore, studies using *E. coli* O157:H7 inocula were undertaken in neonatal lambs. Six-day old colostrum-fed (CF) lambs were used, as this represented a reasonable compromise between susceptibility to colonisation by *E. coli* O157:H7 and susceptibility to naturally-acquired infectious diseases of the new-born, which might adversely affect the experiments. Colostrum feeding should provide protection against neonatal infection in a non-gnotobiotic environment without precluding detectable *E. coli* O157:H7 adherence, as both colostrum-deprived and CF neonatal calves developed AE lesions when inoculated orally with *E. coli* O157:H7 (Dean-Nystrom et al. 1997). The primary aim of the studies was to establish in principle whether *E. coli* O157:H7 can associate with the intestinal mucosa in orally inoculated sheep. Therefore, potentially highly susceptible individuals and a high dose inoculum were used, in a short-term experiment.

5.2 Materials and methods

Animal procedures

The housing, inoculation, monitoring and sampling of lambs are detailed in sections 2.6.1 and 2.6.3.1A. Four lambs were used and one lamb was tissue-sampled and euthanased on each day p.i. Alimentary tissue sampling was comprehensive, including the tonsils, small intestine and mesenteric lymph nodes. All alimentary sites sampled, including duodenum and jejunum, were examined by light microscopy.

Bacteriological procedures

The lambs used were screened at four days of age by faeces culture for *E. coli* O157:H7 via IMS, as detailed in Section 2.2.9 although without the preparation of a dilution series before pre-enrichment.

The preparation of inocula is described in Section 2.2.7. A four-strain mixture of Shiga toxin-producing *E. coli* O157:H7 was used, comprising two bovine-derived and two human-derived strains. Candidate strains were screened for established virulence-associated genes (Section 3.3) and for associated phenotypic characteristics (Section 3.5). The selection process is described in Section 3.6, and the shared and variable features of the four strains are summarised in tables 3-L and 3-M respectively.

The recovery and the analysis of bacteria are described in sections 2.2.9, and 2.2.10 respectively. The sites from which samples were cultured were: rumen, distal small intestine (ileum), large intestine, tonsils and mesenteric lymph nodes.

Pathological procedures

Details of tissue fixation and the preparation, staining and examination of LM sections are given in sections 2.7.1and 2.7.2 respectively. For immunostaining (Section 2.7.3), sections were prepared from seven pieces of embedded tissue, comprising caecum from Lamb 5 (12 hours p.i.) and terminal colon and rectum from Lamb 8 (84 hours p.i.). Following examination of tissues by LM, small pieces of tissue were cut out of the paraffin wax blocks and processed for TEM, as detailed in Section 2.7.4.2A. Immunogold staining (Section 2.7.4.3) was performed on sections from one resin-embedded specimen.

5.3 Results

Clinical findings

All lambs remained normal throughout the experiment. Daily rectal temperatures were within the normal range.

Bacteriological findings

Pre-inoculation faeces screening of the lambs did not detect *E. coli* O157:H7. The inoculated mixture provided 9 x 10^8 cfu/animal.

Faeces culture

The inoculated animals had a low and irregular level of excretion at six and 12 hours p.i., and a higher level of excretion of most strains thereafter (Table 5-A). In the later samples, at 60 and 84 hours p.i., the EC157 str^r and EC222 $nal^r rif^r$ strains were detected at lower levels than the two other strains, but no clear pattern can be discerned.

Table 5-A: Logarithmic counts of *E. coli* O157:H7 recovered from rectal swabs and faeces of orally inoculated neonatal lambs

Lamb	5				6				7				8			
Strain	**n**	**r**	**s**	**nr**	**n**	**r**	**s**	**nr**	**n**	**r**	**s**	**nr**	**n**	**r**	**s**	**nr**
6 hours p.i.	–	–	–	–	1	1	–	1	1	1	–	1	4	4	1	4
12 hours p.i.	–	–	–	–	–	–	–	–	1	1	–	–	≥6	≥6	4	≥6
36 hours p.i.					≥6	≥6	≥6	≥6	≥6	≥6	≥6	≥6	≥6	≥6	≥6	≥6
60 hours p.i.									≥6	≥6	2	4	≥6	≥6	4	≥6
84 hours p.i.													≥7	≥7	≥7	–

Lambs 5, 6, 7 and 8 were inoculated with a mixture of 140065 nal^r (**n**), EC218 rif^r (**r**), EC157 str^r (**s**) and EC222 $nal^r rif^r$ (**nr**). Faeces samples (swabs at six and 12 hours, 1 g of faeces thereafter) were taken from all lambs alive at the time points in the table. Values are $\log_{10}$ of the most probable number cfu/g in an ascending 10-fold dilution series. – indicates *E. coli* O157:H7 not detected.

Tissue and intestinal contents culture

E. coli O157 was isolated from the majority of cultured samples of the gastrointestinal tract (Table 5-B). In all cases high numbers were recovered from the caecum and spiral colon (colon 2). In Lamb 6, high numbers of all inoculated *E. coli* O157 strains were recovered from terminal colon (Colon 3) tissue and contents, despite no such organisms having been cultured from the rectum. Both the terminal colon and rectum were well colonised by *E. coli* O157 in lambs 7 and 8, at 60 and 84 hours p.i. respectively. The ileum of all animals yielded modest numbers of *E. coli* O157, and low numbers were recovered from the rumen. Low numbers of organisms were recovered from the mesenteric lymph nodes of one animal and from the tonsils of three.

Table 5-B: Logarithmic counts of *E. coli* O157:H7 recovered from tissues and intestinal contents of orally inoculated neonatal lambs

Lamb	5 (12 h.p.i.)				6 (36 h.p.i.)				7 (60 h.p.i.)				8 (84 h.p.i.)			
O157:H7 strain	**n**	**r**	**s**	**nr**	**n**	**r**	**s**	**nr**	**n**	**r**	**s**	**nr**	**n**	**r**	**s**	**nr**
Mes. lymph nodes	–	–	–	–	–	–	–	–	1	1	1	1	–	–	–	–
Tonsil	2	2	–	–	2	2	2	2	2	2	2	–	–	–	–	–
Rumen	2	4	2	2	2	2	2	2	2	2	2	2	4	1	–	–
Ileum	4	4	2	4	4	4	2	2	≥6	4	4	1	≥6	≥6	≥6	4
Caecum	≥6#	≥6#	≥6#	≥6#	≥6#	≥6#	4#	≥6#	≥6	≥6	≥6	4	≥6	4	4	2
Colon 2	≥6	≥6	≥6	≥6	≥6	≥6	1	≥6	≥6	≥6	≥6	4	≥6	≥6	4	–
Colon 3 tissue	4	4	2	4	≥6	≥6	–	≥6	≥6	2	–	2	≥6#	≥6#	4#	2#
Colon 3 contents	–	2	–	–	≥6	≥6	–	≥6	≥6	≥6	–	4	≥6#	≥6#	≥6#	4#
Rectum tissue	4	4	–	4	–	–	–	–	≥6	2	–	2	≥6#	4#	2#	–#
Rectum contents	2	2	–	2	–	–	–	–	≥6	≥6	–	≥6	≥6#	≥6#	≥6#	2#

Lambs 5, 6, 7 and 8 were inoculated with a mixture of 140065 nal^r (**n**), EC218 rif^r (**r**), EC157 str^r (**s**) and EC222 nal^r rif^r (**nr**). Samples were collected and cultured as described in Section 2.2.9. Values are $\log_{10}$ of the most probable number cfu/g in an ascending 10-fold dilution series. #: sites where AE lesions were detected histopathologically. h.p.i. = hours post inoculation.

Macroscopic pathological findings

Macroscopic abnormalities were not observed in any tissue. Tissues typically were placed in fixative within one minute of excision from the lamb.

Histopathological findings

Detailed LM observations are presented in Table 5-C. Intestinal tissue preservation was excellent (no appreciable epithelial detachment) in the large intestine and excellent or good (slight epithelial separation with or without minor epithelial loss) in the small intestine.

In the small intestine, mild to focally moderate infiltration of the lamina propria by eosinophils and/or neutrophils was present in the duodenum and ileum of Lamb 5, the jejunum and ileum of Lamb 6, the ileum of Lamb 7, and the jejunum and ileum of Lamb 8. Neutrophils were present in the mucosal epithelium in some sections.

From each sampling site in the large intestine, six to 11 sections were examined. When measured macroscopically, the total length of the mucosal edge of the sections examined from each site was between 75 and 200 mm. Small, focal areas of closely-adherent bacteria resembling AE lesions were seen at the mucosal surface in the caecum of lambs 5 and 6, sampled at 12 and 36 hours p.i., and in the terminal colon and rectum of Lamb 8, sampled at 84 hours p.i. Lesions were not observed in the tissues of Lamb 7. Only seven AE-type lesions were detected in 191 large intestine H&E-stained sections. Each lesion extended over 3 to 15 adjacent enterocytes which usually protruded slightly, either singly or as a group,

above the surrounding mucosal surface (figures 5-a and 5-b). Affected enterocytes often appeared to have rounded up, and some showed condensation of nuclear material. One caecal lesion was accompanied by an overlying clump of neutrophils, some of which appeared to contain bacteria (Figure 5-c). Detachment of a colonised enterocyte from the mucosa was also observed.

Mild to focally moderate infiltration of the lamina propria by eosinophils and neutrophils was present throughout the large intestine in a pattern which varied between animals and which showed no apparent association with the presence of visible AE lesions. Occasionally, neutrophils were seen in the intestinal epithelium. Pathological changes were not observed in sections of rumen, liver, kidney, tonsil, mesenteric lymph node and spleen from any animal.

Immunohistochemical findings

Five of the seven pieces of tissue selected for the preparation of ipx-stained sections were chosen because AE lesions had been observed in H&E-stained sections prepared from the same piece. In view of the low incidence of lesions seen in the H&E-stained sections overall, it was anticipated that these selected pieces of tissue would be most likely to yield sections containing lesions. The two other pieces of tissue which were used were embedded in the same wax blocks as two of the five pieces described above, and so were sectioned and stained alongside them.

None of the five lesions that had previously been identified (in H&E-stained sections) were seen. However, one further lesion was found in a section of rectum from Lamb 8. This piece of tissue had not shown any AE lesion in H&E-stained sections. The adherent bacteria were positively and specifically labelled by the O157 antiserum (Figure 5-d). In a section of caecum from Lamb 5, intestinal contents were seen to contain a large number of specifically-labelled O157 organisms.

Electron microscopy

Pieces of the caecum of Lamb 6 (36 hours p.i.) and the rectum of Lamb 8 (84 hours p.i.) were processed for TEM. These tissue pieces lay directly beneath AE lesions detected in H&E-stained sections taken from the same blocks. Only the lesion seen by LM in the rectum of Lamb 8 was seen in resin-embedded tissue sections. Several bacteria were seen in this lesion, forming typical AE lesions with intimate attachment to the enterocyte plasma membrane, formation of pedestals and effacement of the host cell microvilli (Figure 5-f). There were a few bacteria which were dividing whilst attached to the mucosal surface. The immunogold technique using the O157 antiserum specifically labelled the cell wall of adherent bacteria in the lesion (figures 5-g and 5-h).

5.4 Discussion

When approximately 10^9 cfu of a four-strain mixture of *E. coli* O157:H7 was administered orally to four six-day old conventionally-reared lambs, the animals remained clinically normal throughout the experiment. All inoculated strains were excreted in faeces, with uniformly high excretion at 36 hours p.i., and all strains were recovered from the ileum and caecum of all lambs when one lamb was euthanased at each of 12, 36, 60 and 84 hours p.i. The detection of experimental strains was frequent but variable at other alimentary tract sites. The str^r and the $nal^r rif^r$ strains generally were recovered in lower numbers than either the nal^r or the rif^r strains. Strain differences related to colonisation of the ovine host, or attenuation due to the antibiotic resistance marking, may have contributed to the differences in the ability to recover bacteria.

A four-strain mixture of Shiga toxin-producing *E. coli* O157:H7 was used, comprising two bovine-derived and two human-derived strains, for the reasons outlined in Section 3.6. To avoid the complicating effects of an endogenous *E. coli* O157:H7 flora, lambs and their dams were screened for the excretion of the organism prior to inoculation.

Resources available under category 3 containment permitted four animals to be used, and it was decided to examine one lamb on each day p.i., in order to observe the possible development of adherence over time. The inoculum dose aimed for (10^9 cfu) was chosen to provide a reasonable opportunity for the detection of bacteria-host interactions. The dose-to-bodyweight ratio was similar to that which was used in neonatal calf studies by Dean-Nystrom et al. (1997, 1998) and in a suckling lamb study by Kudva et al. (1995). It was higher than the doses of 10^5 and 10^7 cfu which had proved inadequate to induce reliable excretion of *E. coli* O157:H7 in suckling lambs (Kudva et al. 1995) and young adult cattle (Cray and Moon 1995) respectively. It was anticipated that the size of dose given would not cause such extraneous effects of a high bacterial load as massive Shiga toxin or endotoxin release; such phenomena would be unlikely to occur in the field and might confuse the results if they occurred experimentally. The dose given produced no clinical effects and only sparse lesions. This is consistent with the findings of Cray and Moon (1995), who did not observe any clinical signs following the oral inoculation of calves with 10^{10} cfu of commensal *E. coli*.

Oral inoculation was selected as being the most suitable method for introducing the bacteria to the alimentary tract, since it potentially exposed all structures from the mouth, including the tonsils, to the rectum. The sites selected for culture (rumen, ileum, large intestine, tonsils and mesenteric lymph nodes) were chosen in view of the fact that in one report (Dean-Nystrom et al. 1997) the ileum and large intestine, but not the proximal small intestine, were identified as the preferential sites for *E. coli* O157:H7 AE lesion formation. Other reports also have shown the rumen, ileum and large intestine of ruminants to be

colonised by *E. coli* O157:H7 in preference to the intervening small intestine (Cray and Moon 1995; Brown et al. 1997; Cornick et al. 2000; Tkalcic et al. 2000; Fischer et al. 2001). Whilst a high, sustained level of *E. coli* O157:H7 was seen in the small intestine of calves in one study (Woodward et al. 1999), this was in a gnotobiotic background. Mesenteric lymph nodes and tonsils have been culture-positive in calves inoculated with *E. coli* O157:H7 (Cray and Moon 1995; Woodward et al. 1999).

To obtain tissue samples which were of the highest possible quality for bacteriological and histological examination, the sampling technique employed aimed to fulfil the following criteria:

a) Minimal time between loss of *in vivo* intestinal tissue perfusion and immersion in fixative, as autolysis is rapid in the intestine (Fell 1961).
b) Minimal potential for bacterial cross-contamination, as sensitive bacteriological detection methods were being used to detect potentially low numbers of bacteria in each site.

As the experiments were performed in the Aerobiology Unit apparatus, only one person could manipulate the animal and tissues when sampling, owing to constraints of the equipment (Figure 2-a). For this reason, and to further improve on the tissue quality obtained in a pilot experiment (6/1), tissues were sampled with the lambs under terminal anaesthesia rather than immediately after euthanasia. The bacteriological and histological results, exhibiting generally good tissue preservation and marked variation in bacterial recovery from site to site, indicate that the objectives of the sampling technique were met.

There was poor correlation between culture results for faecal excretion and alimentary tissue samples. For example, at 36 hours p.i., all inoculated strains were detected at high levels in the faeces of Lamb 6, but when nearly contemporaneous (i.e. during the same morning) tissue samples were collected from the same lamb, *E. coli* O157:H7 was not detected in the rectum, despite there being a large number of organisms in the adjacent terminal colon. Some of these unexpected findings may be artefactual, and possible mechanisms for this include: entrapment of IMS beads by debris associated with tissue or intestinal content (this was regularly observed in neat samples), inhibition of bacterium-bead association by substances in the intestinal contents or released from the disrupted tissue, and direct inhibition of *E. coli* O157:H7 growth by substances present in particular parts of the alimentary tract.

Although three inoculated strains were present in the tonsils for up to 60 hours p.i., there was no histological evidence of *E. coli* O157:H7 colonisation of the associated mucosa or tissues. The detection of a low level of all experimental strains in the mesenteric lymph nodes of one lamb, in the absence of histological evidence of colonisation, may represent contamination at the time of sampling. Occasional recovery of an orally inoculated *E. coli*

O157:H7 strain from mesenteric lymph nodes has been noted in other reports (Cray and Moon 1995; Woodward et al. 1999).

AE lesions were detected at 12, 36 and 84 hours p.i. and were confirmed to be associated with *E. coli* O157:H7 by immunolabelling. The restriction of lesions to the large intestine is consistent with the γ-intimin subtype of *E. coli* O157:H7 which, in the piglet, appears to be a significant factor in the site-specificity of AE lesions (Nataro and Kaper 1998). Despite the culture evidence of high numbers (frequently $\geq 10^6$ cfu/g) of *E. coli* O157:H7 in the intestinal lumen, AE lesions were observed in only five out of 197 H&E or ipx stained large intestine sections examined, with a total of eight lesions overall. The identity of the particular strain(s) forming the AE lesions was undetermined. The *E. coli* O157:H7 counts in the locations where AE lesions were detected were all at or above 10^6 cfu/g, a concentration which Dean-Nystrom et al. (1999) has suggested is the lower limit for histopathological detection of such lesions. The lesions, and in one case a demonstrable local inflammatory response (Figure 5-c), were established by 12 hours p.i., and showed no tendency to increase in number and/or size over the following three days.

Sections selected for ipx staining were from blocks which had already provided AE lesions in H&E-stained sections. Furthermore, as the sections for immunostaining were taken from tissue closely adjacent in the wax block to that in the corresponding H&E-stained sections, it was possible that parts of lesions already observed in the H&E-stained sections would also be present in the immunostained sections. This proved not to be the case, as the lesions were too small to span many 4 μm section thicknesses. Indeed, the single immunostained lesion that was found was from a piece of tissue which had not yielded lesions in previous H&E-stained sections.

The AE lesions seen were small and sparse, necessitating LM-targeted tissue retrieval from wax blocks for EM examination, which was necessary to confirm the nature of the lesions. Although the appearance of AE lesions by LM is generally characteristic, other types of adhering *E. coli*, such as ETEC (Figure 5-e), may produce lesions which appear similar by LM and which cannot definitively be categorised until the ultrastructure of the host-bacterium interface is examined with EM. Once lesional material was in a resin block, the advantage of reliably cutting sequential thin sections of the lesion, as opposed to the chance incorporation of a tiny lesion in a 4 μm LM section, was a major reason for attempting to label the AE organisms using the immunogold technique. In the event, both ipx- and immunogold-labelling, with LM and EM respectively, proved successful in the specific detection of *E. coli* O157-associated AE lesions.

The success of the immunogold procedure with tissues which had been extensively processed, firstly to wax infiltration and then to epoxy resin infiltration via post-fixation in osmium tetroxide, may have been in large part due to the stability of the O157

polysaccharide antigen epitopes. It is likely that a protein antigen would have been substantially or entirely denatured by such a procedure. It is fortunate that, given the small and sparse nature of the lesions involved, all four detection and staining techniques attempted (i.e. H&E, ipx, TEM, and immunogold) worked successfully, to locate and characterise the lesions. This range of techniques is valuable, to provide several options for the rigorous characterisation of such elusive lesions.

In the present experiment the attachment of *E. coli* O157:H7 to the ovine mucosa was demonstrated and was shown to be AE in nature. There was no evidence of an alternative mode of *E. coli* O157:H7 attachment to the intestinal mucosa. The lesions in this model appeared to be near the limit of detection by conventional histopathological and immunohistochemical means. Whilst the observed frequency and size of AE lesions is, of necessity, based upon examination of a small proportion of the mucosal surface, it is possible that intimate association between *E. coli* O157:H7 and the ovine intestinal mucosa is in some way less effective than in cattle. In neonatal CD calves inoculated with a single strain of *E. coli* O157:H7 at less than 12 hours of age, extensive AE lesions were observed, and fewer lesions were reported from similarly-inoculated CF calves (Dean-Nystrom et al. 1997). The relative paucity of lesions detected in the present study may therefore partly reflect the protective effect of colostrum. However, even in the CF neonatal calves described above AE lesions covering between 10 and 50 % of the caecal mucosal surface were reported, which is considerably more extensive than in the present study. This difference between the calf and lamb studies may be due to differences between any or all of: host species, host age, dose sizes and inoculum strains. Whether inoculation of younger lambs or more prolonged exposure would lead to establishment of more and/or larger lesions is unknown. However, there was no evidence in the present experiment of an increasing incidence or size of lesions over the 84 hours of the study. The calves reported were inoculated at less than 36 hours of age, in comparison with six days for the lambs. It may be that over the first few days of neonatal life the intestine's resistance to AE lesion formation increases substantially, possibly due to alterations in the mucosa, in motility or in the intestinal flora. The inoculum of *E. coli* O157:H7 given to the neonatal calves was 10^{10} cfu, in contrast to approximately 10^{9} organisms used in the present experiment, although the initial concentration of inoculated organisms in the intestine was probably similar in both cases as there is an approximately tenfold difference in body mass between a neonatal calf and a neonatal lamb. The abilities of the non ovine-derived *E. coli* O157:H7 strains used in this work to colonise the ovine mucosa, when compared with other experimental strains and ovine field strains, are unknown. Finally, a possible effect of inter-strain competition on the formation of AE lesions in the present study cannot be discounted, although *in vitro* tests (Section 3.5.4) did not show any inter-strain effects upon growth of any of the inoculated strains.

In conclusion, the neonatal lamb study demonstrated that in principle *E. coli* O157:H7 will form AE lesions on the ovine large intestinal mucosa, and that extensive examination of tissues may be necessary for the detection of such lesions. This study provided further evidence of a host-bacterium adherence phenomenon, forming a basis with which to assist interpretation of persistence and colonisation characteristics of the same *E. coli* O157:H7 strains in older sheep.

Table 5-C: Histopathological findings in tissues from lambs 5 to 8, inoculated at six days of age with 9 x 10^8 cfu of a four-strain mixture of *E. coli* O157:H7 *Continued on page 151*

	Lamb 5, 12 hours p.i.	Lamb 6, 36 hours p.i.	Lamb 7, 60 hours p.i.	Lamb 8, 84 hours p.i.
Rumen	**(2)** EP. Occasional bacteria on epithelial surface.	**(1)** EP. NAD.	**(1)** EP. NAD	**(1)** EP. NAD
Duodenum	**(2)** EP. Normal villus morphology (V:Cr 1:1) Mod LPC: plasma c, eosins, scattered, focally mod PMN. Focal PMN in epithelium.	**(2)** EP. Normal villus morphology (V:Cr 1.5-2:1). Mod LPC: low eosins, occasional plasma c. Minimal epithelial leucocytes.	**(2)** EP. Normal villus morphology (V:C r 2-3:1). Mod LPC: low eosins and PMN. 1 focus of PMN. Minimal epithelial leucocytes.	**(3)** EP. Normal villus morphology (V:Cr 1:1). Mod LPC: low eosins, scattered plasma c. Minimal epithelial leucocytes.
Jejunum	**(2)** EP. Normal villus morphology. Mod LPC: low-mod eosins, mainly round crypts. Minimal epithelial leucocytes.	**(2)** EP. Normal villus morphology (V:Cr 2-3:1). Mod LPC: mod eosins. Minimal epithelial leucocytes.	**(2)** EP. Normal villus morphology (V:Cr >4:1). Mod LPC: low-mod eosins. Minimal epithelial leucocytes. 1 villus shows necrosis of tip.	**(2)** EP. Villus morphology normal (V:Cr 3:1). Mod LPC: mod eosins. Minimal epithelial leucocytes.
Ileum	**(5)** EP. Villus morphology normal. PP's NAD. Mod LPC: mod eosins, mod PMN, also in lumen. A few PMN in epithelium.	**(5)** EP. Normal villus morphology. (V:Cr 1.5:1) Mod LPC: mod eosins, scattered PMN. Minimal epithelial leucocytes.	**(6)** EP. Normal villus morphology (V:Cr 2:1). Mod LPC: mod eosins round crypts, PMN concentrated over Peyer's patches and crypts. Minimal epithelial leucocytes.	**(6)** EP. Normal villus morphology (V:Cr 3-4:1). Mod LPC: mod eosins. Patchy PMN in epithelium and in crypts. Many bacteria apparently adherent to epithelium. Ipx: Minority of bacteria in 2 non-adherent clusters contain positive O157 bacteria.
Caecum	**(5+3 glut.)** EP. Mod LPC: eosins, mod PMN. Mod PMN in epithelium. Occasional crypt abscesses. AE lesion with adherent PMN. Ipx: not present.	**(6+3 glut.)** EP. Mod LPC: occasional plasma c and eosins. 1 AE lesion. Minimal epithelial leucocytes.	**(5+4 glut.)** EP. Mod LPC: mod eosin, occasional plasma c and multifocal PMN. Minimal epithelial leucocytes. Some adherent bacteria. Ipx: not O157.	**(5+6 glut.)** EP. Mod LPC: sparse eosins and PMN. Low PMN in epithelium. Few adherent bacteria.
Proximal loop of ascending colon ('1')	**(6+5 glut.)** EP. mod LPC: low eosins and PMN. Minimal epithelial leucocytes. Lamina proprial haemorrhage.	**(5+5 glut.)** EP. Mod LPC: sparse eosins and PMN. Minimal epithelial leucocytes.	**(5+6 glut.)** EP. Mod LPC: low PMN, scattered eosins and plasma c. Minimal epithelial leucocytes.	**(5+6 glut.)** EP. Mod LPC: mod plasma c, scattered PMN and eosins. Minimal epithelial leucocytes.

Notes: see page 151.

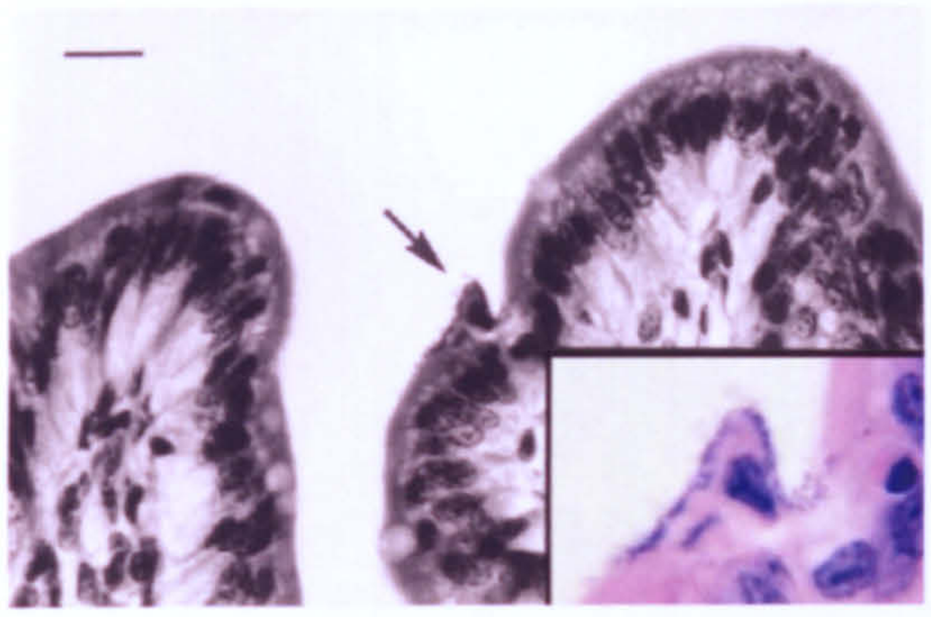
Table 5-C continued

	Lamb 5, 12 hours p.i.	Lamb 6, 36 hours p.i.	Lamb 7, 60 hours p.i.	Lamb 8, 84 hours p.i.
Spiral colon ('2')	**(5+3 glut.)** EP. Mod LPC: eosin, occasional PMN and plasma c. Minimal epithelial leucocytes.	**(5+6 glut.)** EP. Mod LPC: low eosins and plasma c. A few mononuclear cells in epithelium.	**(5+5 glut.)** EP. Mod LPC: scattered eosins and PMN, plus foci of PMN with epithelial transmigration.	**(7+4 glut.)** EP. Mod LPC: low, diffuse eosins. Minimal epithelial leucocytes. Scattered adherent bacteria.
Terminal colon ('3')	**(4+2 glut.)** EP. Mod LPC: occasional eosin and PMN. Epithelium locally ragged. Minimal epithelial leucocytes.	**(5+3 glut.)** EP. Low LPC: rare plasma c and eosins. Minimal epithelial leucocytes.	**(5+6 glut.)** EP. Low LPC: occasional eosins and PMN. Multifocal epithelial PMN. Submucosal blood vessels show margination and extravasation of PMN.	**(6+5 glut.)** EP. Mod LPC: occasional eosins and plasma c. Minimal epithelial leucocytes. 2 AE lesions. Ipx: not present.
Rectum	**(5+4 glut.)** EP. Mod LPC: mod PMN, often necrosing, occasional plasma c. Low, patchy PMN in epithelium.	**(5+3 glut.)** EP. Mod LPC: rare plasma c and eosins. Minimal epithelial leucocytes.	**(5+6 glut.)** EP. Low LPC: scattered PMN plus foci. Minimal epithelial leucocytes. Bacteria evident in some crypts - not adherent.	**(5+5 glut.)** EP. Low LPC: occasional plasma c, eosins and PMN. Minimal epithelial leucocytes. 3 AE lesions (H&E) plus one O157 positive ipx AE lesion.
Mesenteric lymph nodes	NAD	Diffuse, low eosins and PMN. Fresh (surgical) haemorrhage.	Diffuse eosinophils and PMN in 2/3	Scattered eosins and PMN.
Tonsil	Neutrophilic cryptitis	Neutrophilic cryptitis.	Low, diffuse eosinophils.	NAD
Liver Kidney Spleen	Scattered brown pigment in hepatocytes. Siderophages in portal tracts.	Congested spleen. Granular hepatocyte cytoplasm.	NAD	Congested spleen.

Notes: Number of sections examined per site are given in bold parentheses for each intestinal site.
Villus morphology and lymphocyte populations in the lamina propria are normal unless stated otherwise.
Blue text refers to tissue preservation. Red and olive text refers to attached bacteria and to ipx stains respectively.

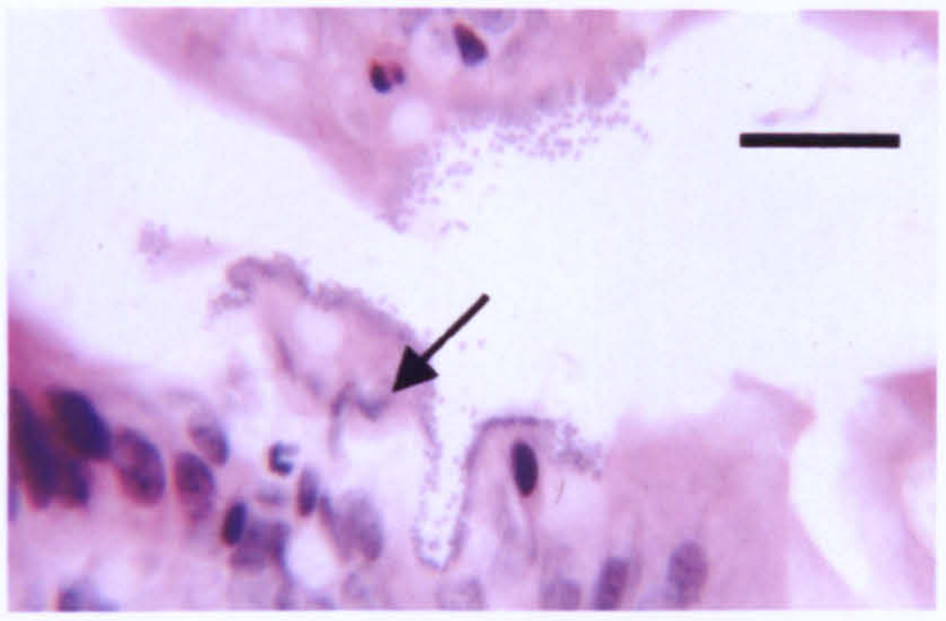
AE: attaching-effacing. **Eosin(s)**: eosinophil(s). **EP / GP**: excellent / good preservation. **Glut**: glutaraldehyde-fixed material (generally smaller sections).
Ipx / ipx: immunoperoxidase stain. **Low / mod**: low / moderate (density). **LPC**: lamina propria cellularity. **PMN**: polymorphonuclear neutrophils.
RBC: Red blood corpuscles. **NAD**: no abnormalities detected. **V:Cr**: villus:crypt ratio.

Figure 5-a: Attaching-effacing lesion



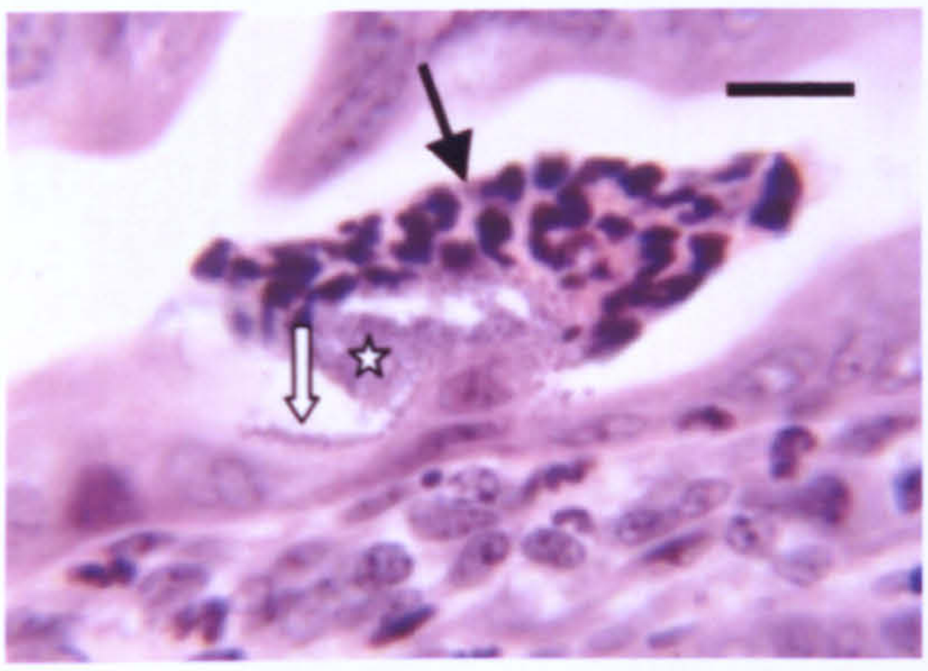
Rectum, Lamb 8 (84 hours p.i.). Affected enterocytes (arrow) protrude from the mucosal surface. Closely-adherent bacteria are evident at higher magnification (inset). H&E, bar = 60 μm.

Figure 5-b: Attaching-effacing lesion



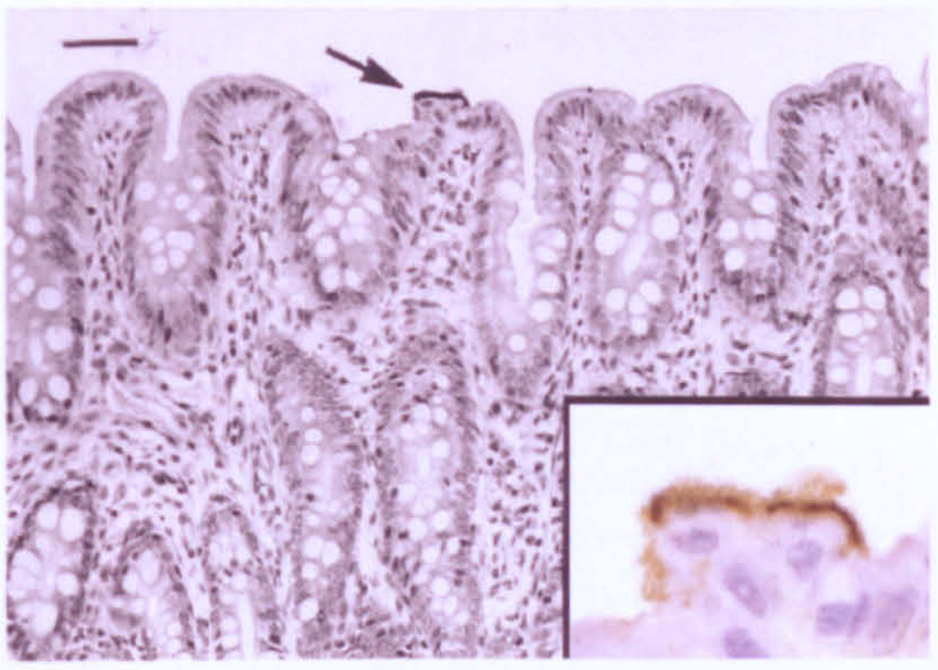
Rectum, Lamb 8 (84 hours p.i.). One of the larger lesions seen. Enterocytes are colonised on either side of a mucosal cleft. Some mucosal cells appear to be degenerate and detaching (arrow). H&E, bar = 20 μm.

Figure 5-c: Attaching-effacing lesion with inflammatory cells



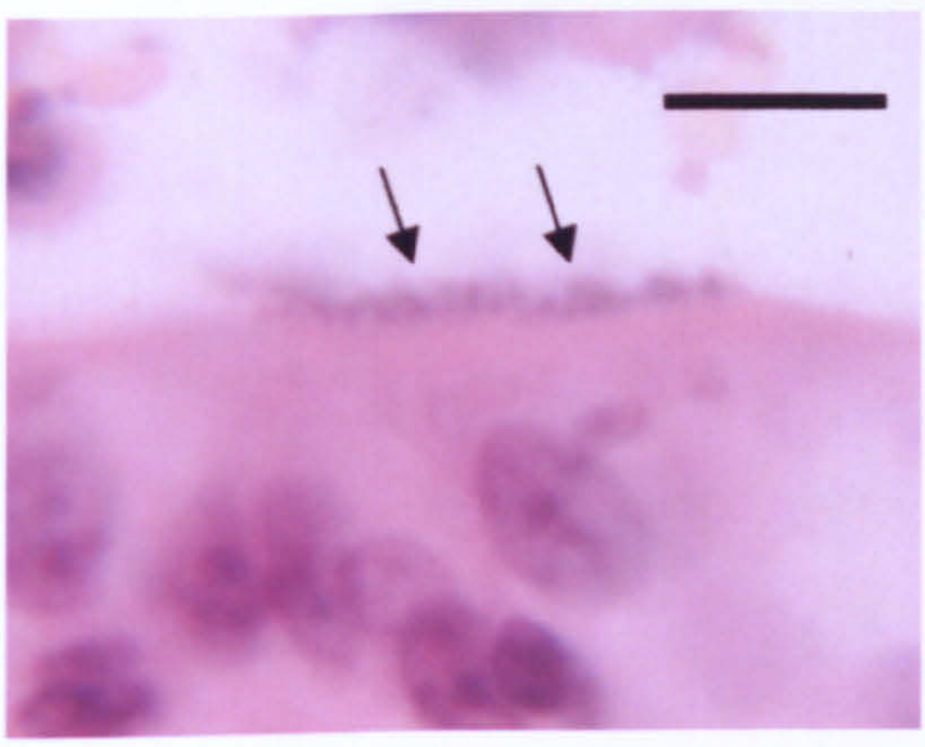
Caecum, Lamb 5 (12 hours p.i.). A mass of polymorphonuclear inflammatory cells (black arrow) overlie a bacterial mass (asterisk) associated with intimately attached bacteria (white arrow). H&E, bar = 16 μm.

Figure 5-d: Immunoperoxidase-stained attaching-effacing lesion



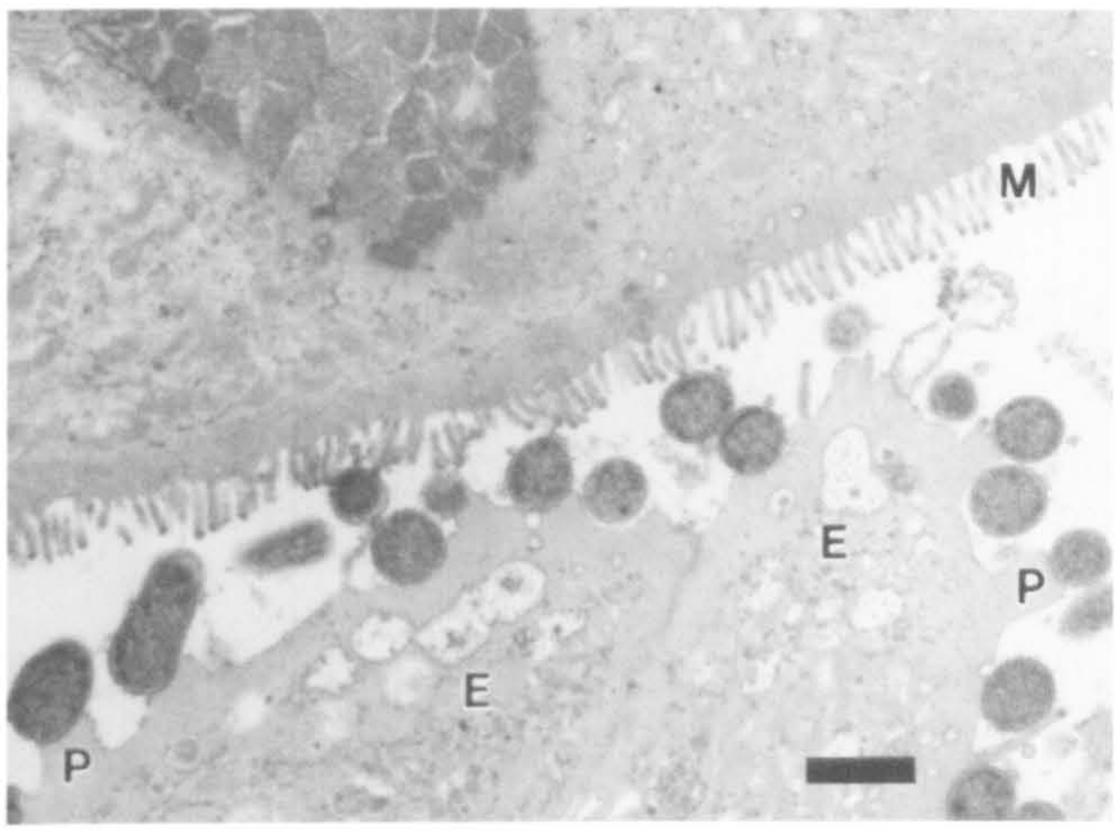
Rectum, Lamb 8 (84 hours p.i.). The lesion protrudes from the mucosal surface. Specifically-labelled *E. coli* cells are evident at higher magnification (inset). O157 ipx, bar = 60 μm.

Figure 5-e: Enterotoxigenic *E. coli*



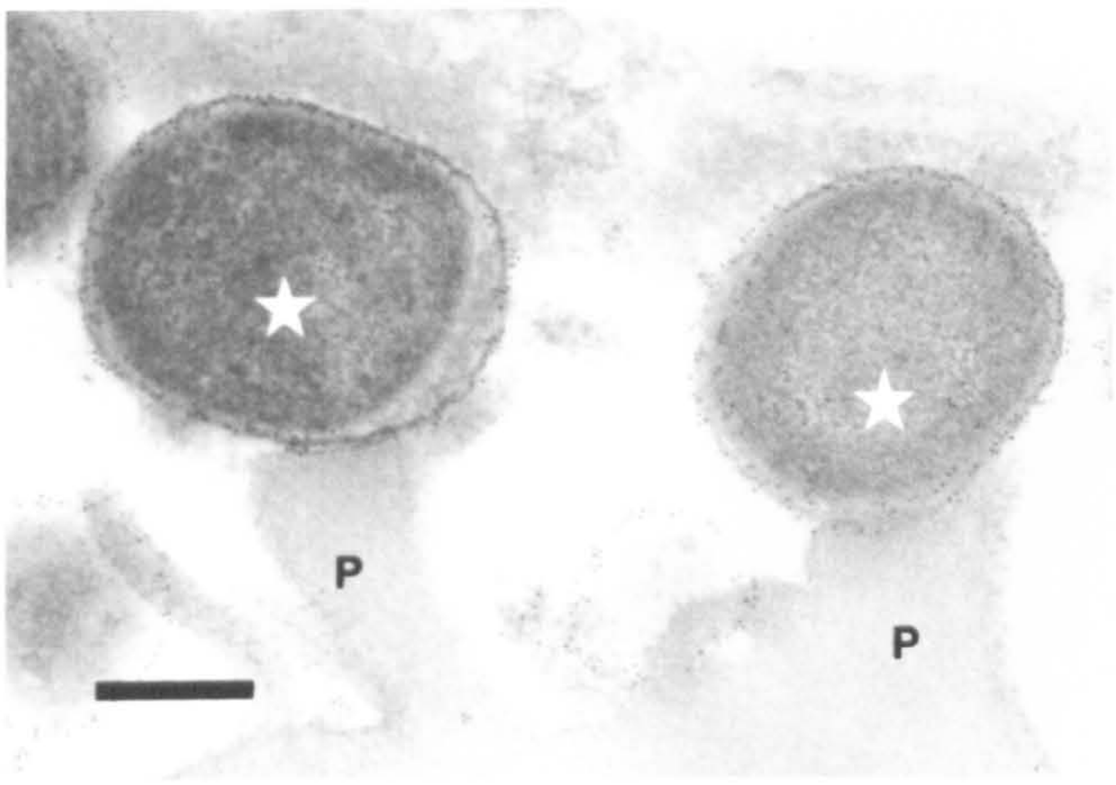
E. coli O101, calf, distal small intestine. Bacteria (arrows) are attached closely to the ileal mucosa. The precise nature of the attachment is not evident under the light microscope. H&E, bar = 10 μm. Section material courtesy of Dr G. R. Pearson.

Figure 5-f: Attaching-effacing lesion



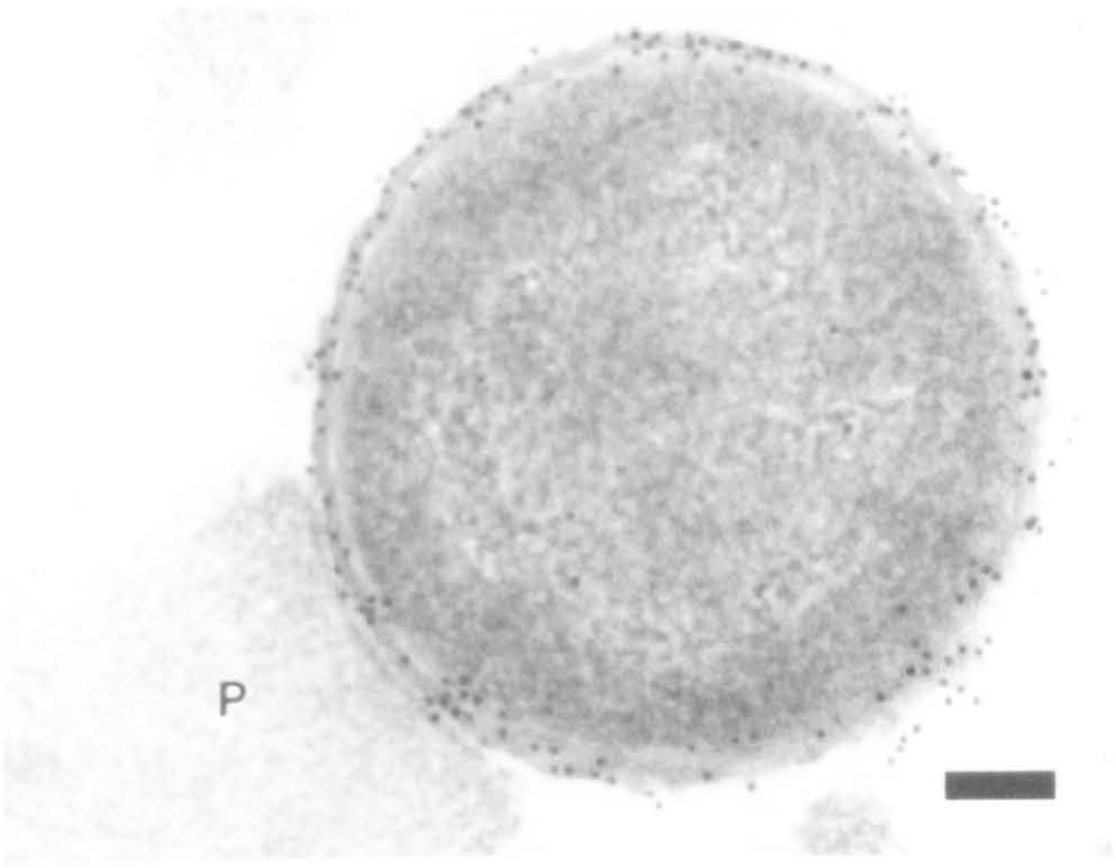
Rectum, Lamb 8 (84 hours p.i., same lesion as Figure 5-a). Bacteria adhere intimately to the enterocyte (E) surface. Microvilli are effaced and some bacteria are on pedestals (P). A normal microvillous border (M) is present on uncolonised adjacent enterocytes. Bar = 1 μm.

Figure 5-g: Immunogold-labelled attaching-effacing lesion



Rectum, Lamb 8 (84 hours p.i.). Gold-labelled *E. coli* O157 organisms (asterisks) are intimately adherent to the apical surfaces of host cell pedestals (P). Bar = 300 nm.

Figure 5-h: Immunogold-labelled *E. coli* O157



Rectum, Lamb 8 (84 hours p.i.). A bacterium is intimately attached to a pedestal (P). Gold particles are localised to the bacterial cell membrane. Anti-O157 immunogold label, bar = 100 nm

Chapter 6 – WEANED LAMB STUDIES

6.1 Introduction

The characterisation of factors important in the persistence of *E. coli* O157:H7 in ruminants is likely to be aided by suitable animal models of persistent colonisation. These models may be used to: characterise the persistence phenomenon using detailed longitudinal studies; look for specific events (such as adherence) at the host-bacterium interface; evaluate the effects of different diets or feed additives; determine the effects of bacterium or host modification, including immune responses, upon persistence. Where persistence specifically is being investigated an inoculated whole-animal approach is likely to prove suitable, as it may permit the interaction of factors known and unknown over an extended time period.

Ruminants inoculated orally or by stomach tube with *E. coli* O157:H7 have been used by several workers in persistence studies. Most reports concern cattle (Cray and Moon 1995; Brown et al. 1997; Cray et al. 1998; Harmon et al. 1999; Hovde et al. 1999; Sanderson et al. 1999; Akiba et al. 2000; Buchko et al. 2000a; Magnuson et al. 2000; Tkalcic et al. 2000; Wray et al. 2000; Besser et al. 2001; Grauke et al. 2002; Naylor et al. 2003), fewer involve sheep (Kudva et al. 1995; Kudva et al. 1997b; Cornick et al. 2000; Lema et al. 2001; Cornick et al. 2002; Grauke et al. 2002) or deer (Fischer et al. 2001). Sheep were used as the model ruminant in the present studies because of their association with the exposure of the human population to *E. coli* O157:H7. They are a species in which natural excretion of the organism has been documented (Kudva et al. 1996; Chapman et al. 1997; Kudva et al. 1997a; Heuvelink et al. 1998a; Chapman 2000b; Synge and Paiba 2000), and there are reports of *E. coli* O157:H7 in sheep meat and milk products (Doyle and Schoeni 1987; Rubini et al. 1999; Chapman et al. 2000b) and of human *E. coli* O157:H7 disease associated with exposure to sheep (Chapman 2000b) and sheep faeces (Ogden et al. 2001). There are six reported studies, detailed in Section 1.4.2.7, of weaned sheep experimentally inoculated with *E. coli* O157:H7. In none of these, using total *E. coli* O157:H7 doses of up to 2×10^{10} cfu per animal, were adverse clinical signs reported.

The aim of the present studies was to use orally inoculated sheep as a model for investigating the persistence of *E. coli* O157:H7 in ruminants, in order to provide data on possible mechanisms of persistence, and on mucosal adherence in particular. The intestinal loop and neonatal lamb studies reported in chapters 4 and 5 were allied experiments in which the AE capability of *E. coli* O157:H7, in inoculated ovine colon loops and orally inoculated neonatal lambs respectively, were investigated. Oral inoculation was selected as being the most suitable method for introducing the bacteria to the alimentary tract in the present

weaned lamb persistence studies, since potentially it exposes to the inoculated bacteria all structures from the mouth (including the tonsils) to the rectum.

An initial pilot experiment at VLA Weybridge (Experiment 6/1) used six-month old lambs to investigate the distribution and maintenance within the alimentary tract of orally inoculated *E. coli* O157:H7 from a four-strain mixture (discussed in Section 3.6), and to seek evidence of bacterial adherence to the intestinal mucosa. In addition the procedures of: inoculum preparation and administration, clean and rapid tissue sampling, and sensitive semi-quantitative bacterial culture from tissues were evaluated.

Due to the availability of experimental resources, subsequent experiments were performed at Langford, using the Aerobiology Unit facilities. Two of these (experiments 6/2 and 6/3) examined the persistence characteristics of strains in the four-strain mixture, and sought evidence for sites and mechanisms of persistence. They were therefore longer-term experiments than 6/1, extending over three (Experiment 6/3) or four (Experiment 6/2) weeks, and incorporating faeces sampling in the protocols.

A final experiment (6/4) used a non Shiga toxin-producing *E. coli* O157:H7 strain, NCTC 12900. The aim of this experiment was to evaluate further the suitability of the NCTC 12900 strain, a category 2 pathogen and therefore potentially of particular value for experimental handling, for use in persistence studies. The capacity for persistent excretion by weaned sheep of this strain over several weeks had been demonstrated previously at VLA Weybridge, by faecal excretion for up to 48 days p.i. (LaRagione, R., personal communication). For this experiment the wild-type strain was used in preference to an antibiotic resistance marked derivative, as a rifampicin-resistant strain had been shown to be markedly attenuated in respect of AE capability *in vitro* (Best, A., personal communication). Experiment 6/4 was done in the category 3 Aerobiology Unit apparatus despite its category 2 status because the experimental set-up in this system had proved successful and relatively straightforward to run. The sampling time points for this experiment were selected to include potential early events in establishing persistence.

6.2 Materials and methods

6.2.1 Six-month old lambs

Animal procedures

The study was performed at VLA Weybridge, where a post mortem room with category 3 containment was available. Animals were inoculated orally and then sampled for histopathological and bacteriological examination, using a protocol of euthanasia followed by rapid sampling to achieve good intestinal tissue preservation. Sections 2.6.1 and 2.6.3.1C

describe housing, inoculation and *post mortem* sampling. Samples were taken at four time points over two weeks, with an emphasis on the early post-inoculation period (one and three days p.i.).

Bacteriological procedures

The pre-inoculation screening of animals for excretion of *E. coli* O157:H7 is described in Section 2.2.9. A four-strain inoculum, detailed in Section 3.6, was used. The dose aimed for was a total of 10^9 cfu per animal. Preparation of the inoculum and the recovery and analysis of bacteria are described in sections 2.2.7, 2.2.9 and 2.2.10 respectively. As two animals were being sampled, sequentially, on each of four days, the bacteriology samples from the second animal to be euthanased on each occasion, i.e. animals 12, 14, 16 and 18 (Table 6-A), were frozen as described in Section 2.2.9 for culture the following day.

Pathological procedures

The fixation, sectioning and staining of tissues for light microscopic examination are described in sections 2.7.1, 2.7.2, and 2.7.3.

6.2.2 Six- to 11-week old lambs

Animal procedures

Two experiments using STEC *E. coli* O157:H7 inocula (experiments 6/2 and 6/3, using mixed and a single-strain inocula respectively) were performed, using four inoculated animals each (designated 19 to 22, and 23 to 26 respectively). Sections 2.6.1 and 2.6.3.1B detail housing, inoculation and sampling under terminal anaesthesia. Two uninoculated 10-week old animals (27 and 28), from the same group from which animals in Experiment 6/3 were taken, were euthanased for histopathological control tissues.

A further experiment (6/4) used a non Shiga toxin-producing *E. coli* O157:H7 (Strain NCTC 12900) inoculated orally into four lambs (designated 29 to 32) housed in the Aerobiology Unit category 3 containment apparatus. Tissue samples were taken from two seven-week old animals (33 and 34) from the same group under terminal anaesthesia, to provide control histopathological tissues. Details of housing, inoculation and sampling are given in sections 2.6.1 and 2.6.3.1B. All animals were examined twice daily, but rectal temperature monitoring was not performed (no deviation from normal had been observed in the previous experiments 6/1, 6/2 and 6/3).

Bacteriological procedures

Pre-inoculation screening of animals for excretion of *E. coli* O157:H7 is described in Section 2.2.9. Details of inoculum preparation are given in Section 2.2.7, with methods (a)

and (c) being used for experiments 6/3 and 6/4 respectively. The culture and analysis of bacteria from experimental samples are detailed in sections 2.2.9, and 2.2.10 respectively. During Experiment 6/4, samples of rectal contents collected at six days p.i. from Lamb 23 were submitted to the University of Bristol School of Veterinary Science bacteriology department to screen for *Salmonella* and *Campylobacter* by the laboratory's standard cultural methods.

In Experiment 6/3, in addition to the standard pre-enrichment / IMS procedure for bacterial recovery, for some samples non-IMS culture was performed in parallel. Two methods of non-IMS culture on CHROMagar O157 supplemented with nal (15 ↘g/ml) were used. Both techniques involved spread-plating an aliquot of each of the neat primary preparations and the 10-, 10^3- and 10^5-fold dilutions (Section 2.2.9) directly on to the CHROMagar. One technique was a TVC, using a 20 µl aliquot of each mixture prior to pre-enrichment incubation. The colonies with an *E. coli* O157:H7 morphology and colour resulting from overnight incubation were counted and a TVC was derived. The other technique used a 100 µl aliquot of the pre-enriched mixtures following a six hour incubation. As the mixture was incubated before subculture, the results were recorded as a most probable number estimate, in a 10- and 100- fold series, based upon the presence or absence of *E. coli* O157:H7 following overnight incubation of the plates. The TVC was performed on most faeces samples, whilst the latter technique was performed for the tissue and intestinal contents samples, in Lamb 25 only.

In Experiment 6/3, a non-*E. coli* O157:H7 isolate, recovered via the non-IMS pre-enriched technique, was serotyped (Section 2.5.1) and genotyped in respect of *eaeA*, *stx1*, *stx2*, *hly*, *espP*, *etpD*, *katP* and *bfpA* genes (Section 2.3).

Pathological procedures

The fixation, sectioning and staining of tissues for light microscopic examination are described in sections 2.7.1, 2.7.2, and 2.7.3. Electron microscopy, used in experiments 6/3 and 6/4, was performed as described in Section 2.7.4.2A. In Experiment 6/4 a 'pop-off' technique (Section 2.7.4.2B) was also used, for retrieving microscopic lesions from LM slide sections for ultramicrotomy and TEM.

6.3 Results

6.3.1 Six-month old lambs (Experiment 6/1)

Clinical findings

All animals remained clinically normal throughout the experiment and their faeces were firm to solid with no evidence of diarrhoea or blood. There was no loss of appetite.

Bacteriological findings

E. coli O157:H7 was not detected from any animal on either of two sampling occasions, four and two weeks prior to inoculation. A total dose of 9×10^8 cfu of the four-strain mixture was given to each animal.

The results from tissue samples taken *post mortem* from each inoculated animal are summarised in Figure 6-a. At one day p.i., inoculum strains were recovered from a wide range of sites (all anatomical locations sampled, including tonsil, rumen and small intestine) from both animals. However, isolation was variable in respect both of the positive sites in a particular animal and of the experimental strains recovered at a particular site. Beyond one day p.i., inoculated strains were not recovered from sites other than the caecum and colon. A low level of three of the inoculum strains was detected in one animal at three days p.i. Neither animal sampled at seven days p.i. yielded any of the strains. A single strain (140065 nal^r) was detected at 15 days p.i., at a low density in the caecum and colon, but not the rectum, of animal 18.

Macroscopic pathological findings

There was evidence of endoparasitism, with a localised mural haemorrhage associated with tapeworm seen in the colon of one lamb, euthanased at 15 days p.i. Other lesions were not observed in any animal at any site examined. The time between euthanasia and placing all small intestinal samples in fixative was less than 6 min, and for large intestinal samples the corresponding time was less than 13 min.

Histopathological findings

Detailed observations are presented in Table 6-A. When control and inoculated lambs are considered together, autolytic changes were observed in 10 out of 20 duodenal and jejunal samples. Samples from the ileum were uniformly well preserved. In the large intestine there were large areas of excellent preservation. However, in some areas early autolytic changes were observed, and in a few sections there was autolytic sloughing of most of the epithelium. The preservation of the mucosa from the terminal colon was uniformly good. In order to ensure examination of a representative portion of the large intestinal mucosa, criteria for

large intestinal sample quality were adopted. Tissues from a particular site were judged to be adequate if there was 80 % or more epithelial coverage in either of the two sections examined, or if both sections demonstrated continuous epithelium covering 50 % or more of the luminal surface. On this basis, five large intestine sites (caecum of lambs 10 and 15, proximal ascending colon of Lamb 12 and spiral colon of lambs 11 and 17) failed to yield samples of adequate quality. In general, when two or more sections from the same intestinal site in an animal were compared, the quality of preservation of the sections was similar. However, in two sites (proximal and spiral colon of Lamb 15) this was not the case, as at each site one of two sections was markedly better preserved than the other.

A luminal bacterial microcolony was associated with epithelial necrosis in the caecum of Lamb 17. Close adherence of bacteria to the luminal surface of enterocytes was not seen in any section. Slender organisms were seen in the crypts of the caecum and colon of three inoculated animals. These were Gram-negative, were shown to be spiral and argyrophilic by the Warthin-Starry silver stain and therefore resembled *Campylobacter* or *Helicobacter* organisms (Figure 6-b). Dense accumulations of neutrophils were present in a few caecal crypts of one animal. Scattered, microscopic foci of necrosis were seen in the large intestinal lamina propria of two inoculated animals (11 and 14). A microscopic focus of epithelial necrosis and sloughing with localised acute inflammation, but without associated bacteria, was seen in the rectum of one inoculated animal (14). The tonsils of inoculated animals frequently showed exudation of neutrophils into crypts around entrapped vegetable matter. Several focal inflammatory and necrotic lesions were seen in the pharyngeal and crypt epithelium. Occasionally these had associated bacteria in the tissue, which in Lamb 18 were shown to be Gram-negative.

Changes which were observed in both control and inoculated animals included: generalised mild blunting, fusion and atrophy of villi in the small intestine and a mild to moderate, diffuse eosinophilic inflammatory infiltrate within the lamina propria of the small and large intestine. Encysted nematodes were present in the mucosa of the duodenum and caecum of some animals. Mucosal structures, resembling coccidian parasites of the genus *Globidium*, and luminal tapeworm segments were also seen.

In the liver, mild chronic portal tract inflammation was seen commonly in both control and inoculated animals. The mesenteric lymph nodes, spleen and kidneys of all animals had a normal appearance.

Immunohistochemical findings

The O157 antiserum proved to be sensitive and specific for *E. coli* O157 over a broad range of dilutions when used to stain control sections of cultured *E. coli* O157. Similar sections of cultured *E. coli* O26, used as negative controls, did not stain with the antiserum.

Control stains using O26 antiserum and normal rabbit serum on O157 organisms were also negative. The bacterial microcolony associated with epithelial necrosis in the caecum of Lamb 17 and Gram negative bacteria in the tonsils of Lamb 18 did not stain with the O157 antiserum.

6.3.2 Six- to 11-week old lambs

6.3.2.1 Four-strain inoculum (Experiment 6/2)

Clinical findings

All lambs remained clinically normal throughout and their faeces were firm, with no evidence of diarrhoea or blood. There was no evidence of pyrexia, loss of appetite or dehydration.

Bacteriological findings

E. coli O157:H7 was not isolated from any of the pre-inoculation faeces samples from any animal. A total dose of 5×10^8 cfu of the four-strain mixture was given to each animal.

The results of daily faeces culture and culture of intestinal samples taken under terminal anaesthesia are summarised in Figure 6-c. All lambs excreted all four inoculum strains for at least four days p.i. Detectable excretion of three of the strains (EC157 str^r, EC222 $nal^r rif^r$, and EC218 rif^r) ceased between four and nine days p.i. in all lambs; in each animal this happened at least one day before euthanasia of the lamb. Strain 140065 nal^r was excreted, at concentrations between 10^1 and 10^6 cfu/g, by Lamb 19 until euthanasia at eight days p.i., by Lamb 20 until 12 days p.i., and by lambs 21 and 22 until euthanasia at 21 and 28 days p.i. respectively. There was no evidence of intermittent excretion of any strain. Of the four inoculated strains, only strain 140065 nal^r was recovered from tissues, in low numbers from the caecum and spiral colon of Lamb 19 at eight days p.i.

Macroscopic pathological findings

Macroscopic abnormalities were not seen in any inoculated lamb. The two control lambs (27 and 28) were clinically normal at the time of euthanasia. Their mesenteric lymph nodes were prominent and were of even shape and consistency. The contents of the terminal colon and rectum of the control lambs were in one case solid and in pelleted form, and in the other a semi-solid unpelleted mass.

Tissues typically were placed in fixative within one minute of excision from the lambs.

Histopathological findings

Detailed observations are presented in Table 6-B. From the four inoculated animals, eight ruminal, 40 small intestinal and 98 large intestinal sections were examined. Preservation of the tissues generally was excellent. Occasionally slight autolysis was evident. Features of the small intestine included a mild or moderate, diffuse eosinophil infiltrate in the lamina propria of all animals, including controls, and a localised acute inflammation, with neutrophil infiltration, in the duodenal lamina propria of Lamb 19. Similarly, eosinophilic inflammation was present in many large intestinal samples from both inoculated and control animals. Luminal debris, including bacteria, was commonly seen adhering to the mucosa of the small and large intestines, including in the control lambs. Rod-shaped bacteria were seen in the tonsils of lambs 19, 20 and 21. Significant pathological changes were not seen in other non-intestinal tissues. Slender organisms resembling *Campylobacter* or *Helicobacter* were seen in some large intestinal crypts of the control animals.

Immunohistochemical findings

Representative sections of intestinal sites where bacteria were seen adhering to the mucosa were immunostained for the presence of O157 antigen (Table 6-B). Positive staining of bacteria was not observed. Bacteria in the tonsils of lambs 19 and 20 also were not labelled by the O157 antiserum.

6.3.2.2 Single-strain Shiga toxin-producing inoculum (Experiment 6/3)

Clinical findings

Lamb 23 had mild diarrhoea at 5 days p.i. and soft faeces on day 6 p.i. when it was euthanased. Lambs 24 to 26 had soft faeces from day 5 p.i. until they were euthanased. There was no loss of appetite or evidence of dehydration in any of the inoculated lambs.

Bacteriological findings

E. coli O157:H7 was not isolated from any of the pre-inoculation faeces samples from any animal. Samples tested for *Salmonella* and *Campylobacter*, in view of the clinical signs manifested by Lamb 23 ('Clinical findings' above), proved negative for both organisms. A total dose of 9×10^8 cfu of strain 140065 nal^r was given to each animal.

The bacteriological findings, using both IMS and direct cultural methods, in respect of *E. coli* O157:H7 are summarised in Figure 6-d. Strain 140065 nal^r was recovered from daily faecal samples from all lambs until day 6 p.i., when Lamb 23 was euthanased. Bacteria were recovered (10^1 to $\geq 10^3$ cfu/g tissue) throughout the alimentary tract from rumen to rectum from this lamb. In Lamb 24, variable excretion of strain 140065 nal^r was observed. It was not

isolated from faeces by IMS on days 10, 11, 13 or 17 p.i., or from any tissue sites when the animal was euthanased at day 17 p.i. Lamb 25 excreted the inoculated strain each day until euthanasia on day 18 p.i. On examination of the tissues from this lamb the 140065 nal^r strain was only recovered from the contents of the terminal colon, and only by pre-enriched direct subculture, when IMS was negative. From Lamb 26 strain 140065 nal^r was recovered consistently in high numbers ($\geq 10^6$ cfu/g) from the faeces, and was recovered from all sites between the ileum and rectum inclusive, and in high numbers (10^3 to $\geq 10^5$ cfu/g) from the large intestine.

Where TVC were performed in parallel with the semiquantitative IMS procedure, results generally correlated well, with IMS usually producing lower values. From the colon and rectum of Lamb 25 (Figure 6-d, shaded) a few lilac colonies which resembled *E. coli* O157:H7 were isolated using direct culture of the pre-enriched broths on nal-supplemented CHROMagar O157. However, these colonies proved to be negative for O157 antigen with the latex agglutination test. A representative sample of these bacteria from each location was serotyped (Section 2.5.1) and found to be non-motile *E. coli* O115. Genotyping by PCR revealed that both strains possessed the *eaeA* gene (epsilon subtype) but neither *stx1* nor *stx2* genes (Cookson et al. 2002b). The direct culture method was trialled with Lamb 25 following zero recovery of *E. coli* O157 from the tissue and intestinal contents samples of Lamb 24.

Macroscopic pathological findings

Macroscopic abnormalities were not seen in any lamb. Tissues typically were placed in fixative within one minute of excision from the lambs.

Histopathological findings

Detailed observations are presented in Table 6-C. From the four inoculated animals, a total of eight ruminal, 42 small intestinal and 120 large intestinal sections were examined. Preservation of the intestinal mucosa was generally excellent or good. Mild autolytic changes were present at about half of the sites examined, and comprised detachment and loss of a few epithelial cells from a minority of villus tips in the small intestine, and small areas of epithelial detachment and single epithelial cell loss at the luminal surface in the large intestine. Four sites in the large intestine demonstrated more marked autolysis focally.

The small intestine of the diarrhoeic lamb (Lamb 23) had a typical villus:crypt ratio (length:depth, Section 2.7.2) of 1:1 at all sites, in contrast to that of the control lambs, which ranged between 1:1 and 3:1 from site to site. In Lamb 25, euthanased at 18 days p.i., many AE lesions were seen in the caecum, proximal loop of the ascending colon and rectum. The lesions were small or of moderate size, often covering numerous adjacent enterocytes in a section (Figure 6-e), but did not extend to cover large areas of mucosa. Encysted and luminal

nematodes and small crypt abscesses were present occasionally in some sections from inoculated animals. A bacterial colony was seen in a mesenteric lymph node from one inoculated animal. A single focus of acute biliary tract necrosis, restricted to one vessel, not accompanied by inflammation and of unknown cause, was seen in Lamb 26.

Some features were noted in tissues from both the control and the inoculated animals. In several large and small intestine sections, loosely adherent luminal contents including bacteria were present on the mucosal surface. The distribution of low or moderate numbers of plasma cells, polymorphonuclear neutrophils, and eosinophils was similar between inoculated and control animals. Small numbers of coccidial schizonts and gametocytes were present in the epithelium of the small intestine of inoculated and the large intestine of control animals. The tonsillar crypts of control and inoculated animals contained adventitious vegetable matter with accompanying bacteria and inflammatory cells.

Immunohistochemical findings

Sections of caecum and rectum from Lamb 25 containing AE lesions, plus sections of ileum from Lamb 24 containing loosely adherent luminal bacteria and debris, and sections of tonsil from Lamb 26 were stained for O157 antigen (Table 6-C). Neither the bacteria forming AE lesions in Lamb 25 nor the adherent bacteria in Lamb 24 stained positively for O157. The bacterial colony detected in the H&E-stained section of tonsil from Lamb 26 was not seen in the corresponding immunostained section.

Because *E. coli* O115 had been isolated from the large intestine of Lamb 25, in which the AE lesions were found, further sections of caecum and rectum from this animal were immunostained using an O115 antiserum (VLA *E. coli* polyclonal typing serum). The specificity of this antiserum at a working dilution of 1:500 was established by demonstrating that this did not label control sections of cultured *E. coli* O157 or O26 organisms. All of the AE lesions seen in immunostained sections of Lamb 25 were specifically labelled by this antiserum (Figure 6-f), establishing them to be *E. coli* O115.

Electron microscopic findings

Using the method of targeted retrieval of tissue from a wax block (Section 2.7.4.2A), a bacterial lesion from the caecum of Lamb 25 was examined. The lesion proved to have a typical AE appearance, with bacteria closely adherent to the host cell membrane, and frequently on pedestals, in areas of microvillus effacement (Figure 6-g).

6.3.2.3 Single-strain non Shiga toxin-producing inoculum (Experiment 6/4)

Clinical findings

All lambs appeared clinically normal throughout the experiment.

Bacteriological findings

E. coli O157:H7 was not isolated from any of the pre-inoculation faeces samples from any animal. A total dose of 4×10^9 cfu of strain NCTC 12900 was given to each animal.

Bacteriological findings post-inoculation are summarised in Figure 6-h. Excretion of *E. coli* O157:H7 was undetectable in Lamb 31 at seven days p.i. and in Lamb 32 at nine days p.i. The recovery of O157:H7 organisms from the alimentary tract also decreased with time following inoculation. The organism was recovered from all sites of the alimentary tract of Lamb 29, sampled at one day p.i. Thereafter, fewer sites were positive: the organism was recovered from the large intestine on days 3 (Lamb 30) and 5 (Lamb 31) p.i. but was not on day 9 (Lamb 32). *E. coli* O157:H7 was recovered from the rumen of all animals. The representative lilac and O157 latex-agglutination positive colonies which were subcultured and subjected to the multiplex *eaeA*, *stx1* and *stx2* PCR test all had the NCTC 12900 genotype, being *eaeA* positive and *stx* negative.

The use of O157 IMS and a selective *E. coli* O157:H7 culture medium (CHROMagar O157), reduced the growth on culture plates by other intestinal flora. However the use of a strain which was not antibiotic resistance marked, and the consequent lack of an antibiotic in the culture medium, did result in a substantial growth of non-O157 intestinal flora on some CHROMagar plates at all pre-enrichment dilutions. These colonies were pale blue, or colourless with a blue centre, and were negative in the O157 latex agglutination test. In lambs 31 and 32, a mass of discrete or semi-confluent blue or colourless colonies were present from all pre-enriched dilutions of faeces samples taken on the days of euthanasia, thereby potentially obscuring any *E. coli* O157:H7 which may have been excreted. Furthermore, on some plates from tissue and intestinal contents samples from Lamb 32 (marked 'f' in Figure 6-h) the overgrowth of colourless colonies was also sufficient potentially to obscure lilac *E. coli* O157:H7 colonies. Representative examples of 'blue' and 'colourless' colonies were identified, serotyped (Section 2.5.1) and subjected to a multiplex PCR test for *eaeA* and Shiga toxin genes, by the VLA Weybridge *E. coli* and *Salmonella* serotyping unit. A faeces-derived 'blue' colony from Lamb 32 proved to be an *E. coli* O26:K'60' strain encoding *eaeA* and *stx1*, and two 'colourless' colonies, one from the rectum of Lamb 29 and one from the faeces of Lamb 32, were both non Shiga toxin-producing and encoded *eaeA*, but were untypable for somatic (O) antigen.

Macroscopic pathological findings

Macroscopic lesions were not seen in any lamb. Tissues typically were placed in fixative within one minute of excision from the lambs.

Histopathological findings

Detailed histopathological observations are recorded in Table 6-D. From the inoculated lambs, eight ruminal, 56 small intestinal, and 109 large intestinal sections were examined. The preservation of the intestinal mucosa was excellent or good (i.e. in the latter category some lifting of the epithelium with no or minimal epithelial cell loss was observed) in all sections except one. In this, the jejunum of Lamb 32, more extensive loss of epithelial cells had occurred, in an area which corresponded with a part that had not been opened with scissors before immersion in fixative, and where it was therefore presumed that penetration of the fixative had been delayed.

Numerous (up to 100) AE-type lesions, were observed in the caecum and colon of lambs 29, 31 and 32, euthanased at 1, 7 and 9 days p.i. respectively. Lesions were small or of moderate size, multifocal, and covered between one and 20 adjacent enterocytes within a section. No areas of coalescing or confluent lesions were seen. AE lesions were detected only in those lambs (29, 31 and 32) from which colourless bacterial colonies (Section 6.3.2.3) were isolated.

Sections from the control and inoculated animals had similar features, in particular the villus morphology and the mononuclear and polymorphonuclear cell populations in the intestinal epithelium and lamina propria were similar. In both groups of animals the following features were identified: coccidial schizonts and gametocytes in the small and large intestinal epithelium, inflammatory debris in a few crypts, luminal debris and bacteria loosely adherent to the mucosa, and occasionally nematode worms on or in the mucosa. In the ileum and caecum of Lamb 32, there were foci of polymorphonuclear neutrophils in the lamina propria, extending into the intestinal lumen and associated with bacterial colonies in the lumen (ileum) and lamina propria (caecum).

Immunohistochemical findings

Detailed ipx results are included in Table 6-D. Pairs of sections from all four inoculated lambs were stained for O157 and O115 antigen, using each antiserum as a negative control for the other. The slides comprised: Lamb 29, caecum and rectum; Lamb 30, rumen; Lamb 31, caecum and proximal ascending colon; Lamb 32, caecum, proximal ascending and spiral colon and rectum. In addition pairs of sections were immunostained with O26 and O157 antisera, comprising: Lamb 29, caecum and terminal colon; Lamb 31, caecum and proximal ascending colon; Lamb 32, caecum and proximal ascending colon.

E. coli O157 organisms were identified in six AE-type lesions of Lamb 29, in the caecum and rectum. These O157-positive lesions were all small, covering either single, or a few adjacent, enterocytes in a section, and were seen separately from (Figure 6-i), adjacent to (Figure 6-j), and apparently on the edge of (Figure 6-k) non-staining AE type lesions. The non-staining (i.e. non-O157) AE-type lesions formed the large majority of lesions in Lamb 29, and all of the lesions seen in Lambs 31 and 32. Two of the non-O157 lesions seen in Lamb 29 were shown to be formed by *E. coli* O26 by specific immunolabelling (Figure 6-l).

Electron microscopic findings

Attempted examination of the largest observed O157 lesion using the wax block retrieval method proved unsuccessful, as the lesion still was too small to be present in the EM preparation. Therefore the 'pop-off' technique was used to retrieve lesional material from glass microscope slides. Lesions examined in this fashion were: the ipx-stained lesion from the caecum of Lamb 29 in Figure 6-i and a non-immunostained lesion also from the caecum of Lamb 29, which was the negative control slide of a positively-immunolabelled O157 lesion. The former lesion was successfully thin-sectioned and, as shown in Figure 6-m, had a typical AE appearance and corresponded morphologically with the LM appearance. Furthermore, the bacteria exhibited a coating, which was presumed to be diaminobenzidine reaction product precipitate, specifically laid down by the O157 ipx technique. Another, incidental and unattached, bacterium found in the same thin section did not possess such a coating. The second, non-immunolabelled slide also yielded AE lesions in thin sections, which were positively labelled using the immunogold technique with O157 antiserum (Figure 6-n).

6.4 Discussion

A series of experiments were conducted to investigate the persistence and localisation of *E. coli* O157:H7 in sheep, and to seek evidence of an association between the bacterium and host at the intestinal mucosa. In Experiment 6/1, eight six-month old lambs were inoculated with 10^9 cfu of a four-strain mixture of antibiotic resistance labelled STEC O157:H7 strains. In this pilot experiment, the times of tissue sampling were biased towards the early post-inoculation phase, as it was considered that adherence may more readily be seen soon after inoculation, when the intestinal concentration of *E. coli* O157:H7, which is an apparent determinant of the ability to detect AE lesions (Dean-Nystrom et al. 1999), was likely to be highest. As the primary interest in this experiment was the distribution and behaviour of *E. coli* O157:H7 within the animal, faeces sampling was not performed. For reasons of

experimental licenses and the availability of suitable facilities, the technique of rapid tissue sampling *post mortem* was used, rather than an *ante mortem* sampling technique.

In view of the limited persistence and limited histopathological findings in the six-month old animals (Section 6.3.1), subsequent weaned animal experiments at Langford were performed on younger animals, inoculated at approximately seven weeks of age. In each of experiments 6/2 and 6/3, four seven-week old lambs were inoculated with 10^9 cfu of STEC O157:H7; in the former experiment a four-strain mixture was used and in the latter the inoculum was a single, apparently persistent, component strain of the mixture (140065 nal^r). In a final experiment (6/4) a wild-type non Shiga toxin-producing *E. coli* O157:H7 strain (NCTC 12900) was inoculated into four seven-week old lambs at a dose of 6×10^9 cfu per animal. Experiments 6/2, 6/3 and 6/4 were performed in the Aerobiology Unit containment apparatus, with faeces sampling throughout each experiment and tissue sampling under terminal anaesthesia.

Inoculum dose and experimental technique

The inoculum dose aimed for in experiments 6/1, 6/2, and 6/3 (10^9 cfu/animal) was chosen for the reasons discussed in Section 5.4. In the absence of clinical signs attributable to this dose, the inoculum aimed for in Experiment 6/4 was increased to 10^{10} cfu/animal. The terminal anaesthetic tissue sampling technique used in experiments 6/2, 6/3, and 6/4 and the clean sampling technique (Section 2.6.2) appeared to have provided well-preserved, uncontaminated samples, as discussed in Section 5.4. The higher incidence of autolysis, sometimes of an unacceptable degree, in tissues from Experiment 6/1 using a *post mortem* sampling technique, suggests that, by comparison, terminal anaesthesia provides superior quality samples. The observation that occasionally enteric mucosal autolysis may be markedly variable at a single sampling site (Lamb 15) suggests that optimal preservation also requires attention to rapid exposure of the mucosa to fixative, by opening the intestinal sample and dislodging any loosely adherent mucosal contents before leaving the sample to fix.

Following few isolations of inoculated strains from the large intestine of inoculated lambs in experiments 6/1 and 6/2, the tissue samples in experiments 6/3 and 6/4 were expanded to include the intrapelvic rectum to the recto-anal junction.

Clinical signs

Clinical signs, of mild diarrhoea and persistent soft faeces, potentially attributable to the experimental inoculum were seen in only one experiment (6/3, single STEC inoculum). The most severely affected animal appeared to have a degree of villus atrophy in comparison with control animals. Despite the temporal correlation between inoculation and the changes in faecal consistency, the cause of the clinical signs is unclear, as no *E. coli* O157:H7-

associated lesions were observed, and in only one of the four affected animals were any bacterial lesions detected. A luminal toxin may have been responsible in whole or in part for the clinical signs. A contribution by other agents, such as coccidia (also observed in the control animals) cannot be excluded. It is consistent with the reports detailed in the General Introduction (Section 1.4.2.7) that there is no convincing evidence from these studies that STEC O157:H7 is pathogenic in sheep under normal circumstances.

Persistent excretion of inoculated strains

The present studies demonstrate substantial variations, both between *E. coli* O157:H7 strains and between individual host sheep, in respect of persistent excretion. One strain (140065 nal^r) proved to be persistently excreted by most animals during the period up to four weeks p.i., whether inoculated at 1.3×10^8 cfu/animal as part of a mixed inoculum, or at 9×10^8 cfu per animal as the sole inoculum. A marked difference between strain 140065 nal^r and the three other strains in the mixed inoculum is evident from Experiment 6/2, and the bacteriological findings of Experiment 6/1 are also consistent with this. Interpretation of this data suggests that a reasonable working definition of persistence in this model would be excretion beyond two weeks p.i. This is a criterion independently adopted by other workers, examining excretion of *E. coli* O157:H7 by experimetally-inoculated calves (Naylor et al. 2003). The issue of whether any or all of the other three strains in the mixture would prove more persistent when inoculated alone was not addressed by the present studies. Although *in vitro* experiments have shown that all strains grew similarly together in mixed culture (Section 2.5.3), this is unlikely to model accurately their relative growth *in vivo*, when in competition with each other and with the endogenous flora over a period of days. There is no evidence that the less persistent three strains in the faeces-monitored mixed inoculum experiment (6/2) were subsequently excreted after initially becoming undetectable. The marked difference *in vivo* between strains which appear similar *in vitro* (Chapter 3, and discussed below), emphasises the value of using a mixed strain inoculum in initial experiments.

The excretion data from the non Shiga toxin-producing strain NCTC 12900 (Experiment 6/4) suggests a 'non-persistent' phenotype, i.e. excretion did not last for two weeks or more, under similar environmental and dietary conditions as Experiment 6/2, where non-persistent strains were eliminated in less than a fortnight. However, given the animal-to-animal variation observed with the STEC inocula, the number of animals examined beyond the initial post-inoculation phase of three days is too few to support a firm conclusion. Furthermore, the alimentary tract samples from this experiment demonstrated the presence of the organism in animals at seven and nine days p.i. despite no detectable excretion, and work by others (Section 6.1) indicates a capacity for persistence by this strain. Any low-level

excretion of *E. coli* O157:H7 by lambs 31 and 32 at seven and nine days p.i. respectively may have been obscured by overgrowth of the endogenous flora on the selective culture plates.

The *in vivo* persistence behaviour of the inoculated strains may be compared with those characteristics of their phenotypes which were investigated *in vitro*, as detailed in tables 3-L and 3-M and also: quantitative cell association (figures 3-f and 3-j), protein secretion and intimin expression (figures 3-c, 3-d and 3-e), and organic acid resistance (figures 3-l, 3-m and 3-n). However, these comparisons do not reveal any *in vitro* characteristics by which strains designated 'persistent' and 'non-persistent', by the criterion of excretion for less or more than two weeks, may be distinguished. In particular, the degree of strain tolerance to inorganic acid stress, which might be expected to influence abomasal transit, does not appear to affect the time or the concentration at which *E. coli* O157:H7 appears in the faeces following oral inoculation.

Whereas antibiotic resistance marking may attenuate some features of *E. coli* O157:H7 strains (Section 3.7), such as their AE capability (Section 3.5.2C), from the evidence of the present studies and of other reports (Cray and Moon 1995; Brown et al. 1997; Cornick et al. 2000; Cornick et al. 2002) it does not appear uniformly to abolish a capacity for persistence. Because of variations in mechanisms and target genes, any attenuating effect on persistence is likely to vary between antibiotic resistant mutants according to the antibiotic used, the method of resistance labelling (e.g. selection of spontaneous mutants versus gene disruption by insertion of a resistance cassette by allelic exchange) and the use of single- or multiple-resistance mutants. In view of the fact that experimental persistence studies of *E. coli* O157:H7 require sensitive detection of, and discrimination between, inoculum strains in the background of the endogenous intestinal flora, antibiotic resistance marking was a useful tool in the present studies. Problems were encountered where it was not used and the inoculated organism might have been excreted in low numbers, as with Lambs 31 and 32 in Experiment 6/4, as there appears to be the potential for substantial carry-over of non-O157 organisms onto culture media when O157 IMS is used.

The animal-to-animal variation in culture results observed in experiments 6/2 and 6/3, using mixed and single STEC O157:H7 inocula respectively, suggests the existence of animals with 'high', 'medium' and 'low' susceptibilities to persistent excretion of *E. coli* O157:H7. For example lambs 26, 25 and 20 fit these three categories respectively. A similar divergence in the excretion profiles of individuals following oral inoculation of *E. coli* O157:H7 has been suggested for cattle (Grauke et al. 2002), whereby the authors suggested that four groups of animals could be discerned. These were: animals which were not colonised (i.e. did not excrete beyond two days p.i.), those that excreted for less than one week, those that excreted for approximately one month, and those that excreted for two

months or more. Of 18 inoculated animals, there were two, three, nine and four individuals respectively in these categories. Field studies of bovine excretion, for example Conedera et al. (2001), have also identified individuals in a group which apparently excrete the organism more persistently than most of the others. Whether these observations reflect simply a continuous, possibly Normal, distribution of persistence phenotypes, or a genuine discontinuous clustering of phenotypes is unclear, given the limited data available. Statistical analysis of possible distributions would require persistence data from many more animals experimentally inoculated with a persistent strain, under standardised conditions.

As the sheep used in the present experiments were all crossbred, of a similar age, from the same flock and were on the same diet, some factors potentially influencing susceptibility to *E. coli* O157:H7 persistence, such as age, breed and diet, seem unlikely to have significantly affected the results. Genetic variation, previous exposure to *E. coli* O157:H7 or related organisms with consequent immunological or physiological responses, individual endogenous flora and pre-existing alimentary pathological processes are possible underlying causes of variation in animal responses to inoculation with *E. coli* O157:H7. Whilst systemic immune responses to *E. coli* O157:H7 LEE components have been demonstrated in humans (Li et al. 2000), the effects of previous exposure to the organism upon subsequent excretion of orally inoculated *E. coli* O157:H7 are unclear (Sanderson et al. 1999; Wray et al. 2000).

How persistent excretion of *E. coli* O157:H7 occurs in ruminants remains undetermined. One important question is whether ruminants are true reservoirs of the organism, due to persistent colonisation of the alimentary tract, or whether the observed phenomenon of persistence in ruminants is due to continuous recycling of *E. coli* O157:H7 from the animals' environment (Hancock et al. 1998a; 1998b). Evidence that the organism does to some extent establish itself in the intestine includes the observation that the inoculum dose in sheep appears to affect the duration of subsequent excretion (Kudva et al. 1997b; Cornick et al. 2000). Studies examining the location of *E. coli* O157:H7 in persistently-excreting weaned ruminants have consistently found it to be present in the large intestine (Cray and Moon 1995; Brown et al. 1997; Harmon et al. 1999; Cornick et al. 2000; Tkalcic et al. 2000; Fischer et al. 2001). The same workers also have frequently found the organism in the rumen for an extended period after inoculation, although others have not (Buchko et al. 2000a; Grauke et al. 2002). Recently (since completion of the present studies), good evidence has been presented that the recto-anal junction is a principal site of colonisation in many persistently-excreting cattle (Naylor et al. 2003).

A survey in the UK has shown that phage types 4 and 32 of *E. coli* O157:H7 are heavily predominant amongst sheep presented for slaughter, whilst types 2, 8, 21/28 and 32 predominate in bovine-derived samples (Paiba et al. 2002). In the mixed inoculum, only one strain (EC222, phage type 32) was of a 'common' ovine phage type, and this proved to be

non-persistent when inoculated as a double-marked antibiotic resistant mutant in a mixture. Conversely, strain 140065 (phage type 2) was not of a common ovine phage type, yet proved persistent in a single and a mixed inoculum. If susceptibility to, or the carriage of, particular bacteriophages are associated with phenotypic traits (e.g. receptor binding factors) that contribute to the host bias of *E. coli* O157:H7, then the findings in the present studies indicate that these traits are not of overriding importance in determining persistence.

Location of E. coli O157:H7 in the alimentary tract

In the present experiments, the excretion of strains on the day of tissue sampling did not correlate closely with recovery of the same strains from the alimentary tract. In lambs 21 and 22, recovery was not made from any site despite near-contemporaneous faeces samples proving culture-positive. Recoveries from the large intestine of excreting animals were most comprehensive when excretion was at a high concentration, within the range of excretion concentrations detected in the present and previous (Sanderson et al. 1995; Grauke et al. 2002), studies ($\geq 10^5$ cfu/g, lambs 26 and 29). The same sites in lambs excreting at a similarly-defined moderate level, i.e. 10^3 to 10^4 cfu/g, ranged from uniformly positive (lambs 30 and 23 to uniformly negative (Lamb 21). In Lamb 31, *E. coli* O157:H7 was isolated in low numbers from the caecum and rectum, when a faeces sample taken immediately prior to terminal anaesthesia and tissue sampling proved negative.

E. coli O157:H7 was recovered less frequently from the ileum than from the large intestine, but the organism was still found in the ileum at up to 19 days p.i. (Lamb 26). Other sites in the small intestine, where cultured (Experiment 6/4), were positive for *E. coli* O157:H7 only up to three days p.i. The rumen was culture-positive for Shiga toxin-producing *E. coli* O157:H7 only in the early post-inoculation period, of Experiments 6/1 and 6/3. The non Shiga toxin-producing NCTC 12900 strain was detected in the rumen throughout Experiment 6/4 although this experiment only extended to nine days p.i. Interestingly, however, the organism was cultured from the rumen in two lambs (31 and 32) when faecal excretion was not detected, and it was the sole positive site in the latter lamb. None of the 'non-persistent' component strains of the mixed inoculum were recovered from the alimentary tract after three days p.i. In the oldest animals (Experiment 6/1), the only strain recovered from the latest tissue sampling at 15 days p.i. was 140065 nal^r, which was shown in the later experiments to be persistently excreted.

Consideration of the faeces and tissue sample culture data (see 'Sensitivity of detection', below) suggests that the sensitivity of the cultural methods used has affected the pattern which is seen. Nonetheless, from Experiment 6/3 the large intestine (but no particular site within it) and the ileum are the areas from which recovery was made from animals (lambs 25 and 26) which were excreting persistently, i.e. for two weeks or longer. There is no evidence

from the six animals sampled at or after two weeks p.i. that the rumen maintains a persistent *E. coli* O157:H7 population, or that it is constantly re-colonised by the organism from a contaminated environment. The bacteriological findings of the present studies are consistent with those of previous reports of *E. coli* O157:H7 in ruminants (Cray and Moon 1995; Brown et al. 1997; Harmon et al. 1999; Cornick et al. 2000; Tkalcic et al. 2000; Fischer et al. 2001; Grauke et al. 2002) and pigs (Francis et al. 1986; Tzipori et al. 1986) inasmuch as colonisation of the large intestine was seen and there was no evidence of sites of colonisation outside the gastrointestinal tract. The data from the present studies do not point to a specific region within the large intestine for colonisation and do not support suggestions from reports of calf experiments (Cray and Moon 1995; Brown et al. 1997; Harmon et al. 1999; Tkalcic et al. 2000) that the rumen is an important site of persistence. There may be an age effect in the persistence of *E. coli* O157:H7 in the bovine rumen as, by contrast, studies in older (yearling) cattle (Buchko et al. 2000a; Grauke et al. 2002) have not found the rumen to be a site of persistence. Nonetheless, the rumen may be significant at any age in affecting the transit of *E. coli* O157:H7 in sufficient numbers and for a sufficient duration to permit colonisation of the large intestine. The recent propsal that the recto-anal junction is a principal site of colonisation in cattle (Naylor et al. 2003) is consistent with the present bacteriological findings in lambs. The possible role of the terminal large intestine was investigated in the present studies by sampling, culture and histopathological examination of the intrapelvic rectum in experiments 6/3 and 6/4. However, these investigations did not yield evidence of a tropism of *E. coli* O157:H7 for this area.

Sensitivity of detection

The method of culture from experimental animals adopted in the present studies, i.e. non-selective pre-enrichment in BPW followed by IMS and subculture on CHROMagar O157, would be expected to be a sensitive technique for the isolation of *E. coli* O157:H7, based upon previous reports (Section 1.4.2.5). However, these same reports indicate that it may be unreliable in detecting the organism in faeces or intestinal samples at initial concentrations below 100 cfu/g.

The freeze-thaw process before culturing used for some samples in Experiment 6/1 would be expected to lower the sensitivity of the IMS-culture technique, due to the 10-fold dilution in PBS-glycerol before pre-enrichment in BPW (Section 2.2.9) and the potential killing of bacterial cells by the freeze-thaw cycle. There is, however, insufficient data to detect such a trend but the semiquantitative counts obtained from previously frozen samples (on the right side of the columns for one and 15 days p.i. in Figure 6-a) are consequently likely to be under-estimates of the true values.

In Experiment 6/3 a comparative TVC culture technique was used with faeces samples, in addition to the standard pre-enrichment IMS and subculture. This was done to assess the accuracy of the semiquantitative IMS method, and to address the possible problem of a lowered sensitivity of detection associated with entrapment of IMS beads by faecal solids (with consequent loss of the beads in discarded material) in low dilution pre-enrichment broths. The data from Experiment 6/3 (Figure 6-d) suggests that IMS provided semi-quantitative results of acceptable accuracy and, on two occasions (lambs 24 and 25, both at 15 days p.i.), detected excretion of *E. coli* O157:H7 when the direct culture method did not.

It is possible that unexpected negative culture findings, such as in tissue and intestinal contents samples of lambs 21 and 22, were due to inadequate sensitivity of detection. Grauke et al. (2002) reported that culture for *E. coli* O157:H7 using non-IMS enrichment was negative for the large majority of samples from the large intestine of sheep which had been excreting the organism for three to six weeks. The culture methodology reported did not involve IMS and, therefore, it is unlikely that the anomalous findings in the present studies were caused entirely by interference with mechanisms specific to IMS. As an alternative to a problem with culture sensitivity, it may be that the organism persists only in the extreme distal portion of the large intestine, close to or at the anus, and good evidence has emerged subsequent to the present studies that in cattle this is indeed the case (Naylor et al. 2003).

An approach to comparing the sensitivity of detection of IMS with another technique was tried with the intestinal contents samples of one lamb (25), following zero recovery from the intestinal tract of Lamb 24. In this trial, a 100 µl aliquot of the pre-enriched primary preparation was directly subcultured onto a nal-supplemented agar plate, in addition to the standard IMS procedure. Whereas this alternative procedure produced negative results in concordance with the IMS results in the caecum, colon 1 and 2 and rectum, it did yield recovery of the inoculated organism from the terminal colon (Figure 6-d) when IMS failed to do so. By serendipity, it also permitted recovery of an *E. coli* O115 which was coincidentally forming AE lesions in the same animal. Such findings suggest that, when a highly selective solid medium (in this case antibiotic-supplemented CHROMagar O157) can be used, enrichment or pre-enrichment plus direct subculture may be as sensitive or more sensitive a technique than IMS. However, in studies or surveys where there is no antibiotic resistance marking of the *E. coli* O157:H7, the competing flora on the agar plates may well render IMS the most sensitive detection technique.

Association of E. coli O157:H7 and other organisms with the intestinal mucosa

In only one of 20 inoculated weaned sheep (Lamb 29) were O157 antigen-bearing bacteria seen to be adherent to the intestinal mucosa. Despite significant technical obstacles, these bacteria were shown to be forming AE lesions. In this instance the 'pop-off' technique

has demonstrated its potential to characterise lesions which are so small that they may not be present in adjacent sections of the same piece of tissue. The O157 immunogold staining was weak, possibly because of the extensive tissue processing which was required to obtain the lesion in a resin section, and this result should be interpreted with caution. However, when the light and electron microscopical findings are considered together, there is satisfactory evidence of *E. coli* O157 AE lesions in Lamb 29.

The lesions were seen in tissues where the luminal concentration of bacteria was probably in excess of the $10^{5.5}$ cfu/g detection limit suggested by Dean-Nystrom et al. (1999), although interestingly the tissue sample from one affected site (caecum) yielded a bacterial concentration of just 10^{3} cfu. Importantly, these lesions demonstrate for the first time the susceptibility of the intestine, and the large intestine (caecum and rectum) in particular, of weaned lambs to AE adherence by orally administered *E. coli* O157:H7. This complements similar observations made in fasted weaned calves (Dean-Nystrom et al. 1999). There is, however, no evidence currently that such lesions occur in persistently excreting animals. Only one persistently excreting animal in the present studies (Lamb 26) might be expected to have detectable AE lesions if a minimum bacterial concentration of $10^{5.5}$ cfu/g in the large intestine is required. Indirect evidence of a role for intimin, and therefore possibly for AE lesions, in persistent excretion of *E. coli* O157:H7 in ruminants comes from Cornick et al. (2002) in which an intimin-deficient human-derived *E. coli* O157:H7 mutant proved significantly less persistent in cattle and sheep than the parent strain. Nonetheless, it appears that the mutant strain was still excreted by one yearling ox at 60 days p.i. Similarly, Dean-Nystrom et al. (1999) demonstrated that, in comparison with an isogenic intimin-deficient mutant, a wild-type *E. coli* O157:H7 achieved a higher density in the large intestines of orally inoculated weaned calves in a short-term experiment.

An interesting feature in two experiments (6/3 and 6/4) was the detection of non-*E. coli* O157 AE organisms in inoculated lambs. Identification of lesions formed by *E. coli* of serogroups O115 and O26 was possible by the use of immunostaining with selected antisera, following bacteriological isolation of these serogroups. *E. coli* O115 possessing the intimin gene was presumably present in Lamb 25 in high enough numbers that spontaneous nal-resistant mutants were isolated on some nal-supplemented CHROMagar O157 plates, and therefore this was a logical serogroup with which to stain the tissues. Intimin-encoding *E. coli* O26 was isolated from a lamb in Experiment 6/4 which exhibited AE lesions, and so O26 also was a logical choice for a test antiserum on corresponding tissues. The majority of lesions in this latter experiment stained with neither O157 nor O26 antisera and may have been formed by the untypable *eaeA*-positive *E. coli* which formed abundant 'colourless' colonies on the CHROMagar O157 plates. One of the animals with non-O157 AE lesions

(Lamb 25) had soft faeces, in common with the rest of its cohort, but the three other lambs in which similar lesions were observed (29, 31 and 32) showed no clinical abnormalities.

The fact that naturally-acquired field strains of AE organisms, some of which were identified as serogroups of *E. coli*, spontaneously formed AE lesions suggests that sheep may be colonised without clinical disease by non-O157 AEEC, assisted by intimate attachment. Such a mechanism has been hypothesised in the persistent excretion of *E. coli* O157:H7 (Gyles 1998; Cornick et al. 2002), and this hypothesis is supported in the present studies by the demonstration that *E. coli* O157:H7 has a spontaneous AE lesion-forming capacity in weaned lambs. The apparent association between O157 organisms and non-O157 AE lesions in Lamb 29 is of particular interest. Given the scattered nature of the non-O157 lesions and the marked scarcity of O157 AE lesions, the observation of these two phenomena in close proximity, or apparently within the same lesion, suggests a specific association. Mechanisms which might promote co-colonisation by *E. coli* O157:H7 and other AE bacteria include quorum sensing (Sperandio et al. 1999) and the attachment of intimin-bearing *E. coli* O157:H7 to enterocytes bearing Tir secreted by another AE organism. A possible example of the latter occurring *in vitro* has been reported (Ismaili et al. 1998). Conversely, there remains the possibility that other AE bacteria in the intestine may reduce the opportunity for *E. coli* O157:H7 to attach to the intestinal mucosa, for example by inducing specific immunological host responses to LEE proteins. There are no reports of the effect of defined systemic host immune responses upon colonisation by *E. coli* O157:H7, but anti-intimin immunoglobulin supplied to suckling piglets via colostrum was associated with reduced colonisation of the piglets' intestinal tract by orally inoculated *E. coli* O157:H7 (Dean-Nystrom et al. 2002).

Conclusions

The present studies have shown that *E. coli* O157:H7 may be excreted persistently from orally inoculated weaned lambs, but that there is substantial variation in this phenomenon between bacterial strains and between animals. A two-week cut-off between 'persistent' and 'non-persistent' strains is suggested on the basis of the excretion patterns seen.

The correlation between detection in faeces and in alimentary tract samples is poor, which may be due to the sensitivity of the cultural methods used, despite them being generally accepted as sensitive and discriminatory. The large intestine appears to be the primary site of persistence. *E. coli* O157:H7 was shown to be capable of spontaneously forming AE lesions in the large intestine, although not in the context of persistent excretion. The possibility that *E. coli* O157:H7 may be assisted by other AE organisms to colonise the large intestinal mucosa requires further investigation.

Table 6-A: Histopathological findings in Experiment 6/1 (four-strain STEC O157:H7 inoculum in six-month old lambs) *Continued on pages 177 and 178*

Lambs:	Uninoculated controls		1 day p.i.		3 days p.i.		7 days p.i.		15 days p.i.	
	9	10	11	12	13	14	15	16	17	18
Rumen	**(2)** NAD	**(2)** Many bacteria at mucosal surface, no evidence of attachment.	**(2)** NAD	**(2)** NAD	**(2)** Occasional small PMN foci in superficial layers of epithelium.	**(2)** NAD	**(2)** NAD	**(2)** One small focus of PMN infiltration in epithelium.	**(2)** NAD	**(2)** Many bacteria adherent to keratinised epithelium
Duodenum	**(3)** Villi distorted and focally atrophied. Many luminal and encysted nematodes. Few eosins in LP	**(2)** Villi fused and blunted. One encysted nematode. Few eosins in LP.	**(2)** Villi resemble controls. In LP eosins and glob l'cytes focally marked, several PMN foci.	**(2)** Mod autolysis. Villi resemble controls. Plasma cells, eosins plus scattered PMN and mast cells in LP.	**(2)** Villi resemble controls. Eosins concentrated in villus cores, towards tips.	**(2)** Villi appear normal. Few eosins in LP. Mod epithelial lymphocyte transmigration.	**(2)** Villus architecture normal. Eosins concentrated in villus tips.	**(2)** Mod autolysis. Mod eosins in LP. Many encysted and luminal nematodes.	**(2)** Mod autolysis. LP quite cellular, including a few eosins. Several encysted nematodes.	**(2)** Eosins and plasma c. in LP.
Jejunum	**(3)** Variable villus morphology. Few eosins in LP	**(3)** Normal villus morphology. One encysted *Globidium* parasite. Mod eosins in LP	**(2)** Villi broad and stunted. Mod autolytic sloughing of epithelium. Mod eosins in LP	**(2)** Mild autolysis. Variable villus morphology. Mod eosins in LP. Tapeworm in lumen.	**(3)** Villus architecture appears normal. Mod eosins in LP.	**(2)** Mod eosins in LP.	**(2)** Villus architecture normal. Mod eosins in LP.	**(2)** Mod eosins in LP.	**(2)** Mild-moderate autolysis. Many eosins in LP.	**(2)** Mild-moderate autolysis. *Globidium* parasite. Mod eosins in LP.
Ileum	**(2)** Variable villus morphology. More eosins in LP than seen in proximal small intestine.	**(3)** Villi mainly overlie Peyer's patches (altered morphology.). Eosins and a few plasma c in LP.	**(2)** Short villi. Mod eosins in LP, some degranulated. Multifocal mild LP oedema.	**(2)** Broad villi, mainly overlying (normal) Peyer's patches. Mod eosins in LP.	**(2)** Villus morphology normal, cores appear mildly oedematous. Mod eosins in LP.	**(2)** Mod eosins in LP	**(2)** Villi resemble controls. Moderate to large number of eosins in LP.	**(3)** Mod eosins in LP.	**(2)** Many eosins in LP.	**(2)** Many eosins in LP.
Caecum	**(2)** Several encysted nematodes.	**(7)** Patchy autolytic sloughing of epithelium. LP includes eosins, plasma c and PMN.	**(3)** LP and submucosa contain eosins. Surface inflammatory focus with larval nematodes.	**(4)** LP includes eosins and scattered PMN. Two crypts contain neutrophil casts.	**(5)** Autolysis: luminal epithelium mostly sloughed. Few eosins in LP. Slender, spiral bacteria in some crypts, Gram neg, Warthin-Starrey Ag-staining.	**(2)** Mod autolytic epithelial sloughing. Mod eosins in LP. Mononuclear inflammatory band subjacent to muscularis mucosae.	**(8)** Most of luminal epithelium has sloughed. Few eosins in LP.	**(2)** Basophilic masses in lumina of a few crypts. Gram neg, O157 ipx neg. Probably spiral bacteria. Mod eosins in LP.	**(8)** Mild-mod autolysis, but some epithelium ragged with assoc. bacteria. Mixed infiltrate subjacent to muscularis mucosae. Eosins and plasma c abundant in LP. PMN common in LP. Large Gram neg. colony at mucosal surface, associated with necrotic material and damaged epithelium, no clear close adherence. O157 ipx neg.	**(2)** Moderate autolysis. Mod eosins in LP.

Notes: see page 178

Table 6-A continued

Lambs:	Uninoculated controls		1 day p.i.		3 days p.i.		7 days p.i.		15 days p.i.	
	9	10	11	12	13	14	15	16	17	18
Proximal loop of asc. colon ('1')	**(2)** NAD	**(2)** NAD	**(10)** Epithelium sloughing. Eosin and PMN infiltrate in LP. Small, focal LP necrosis.	**(8)** Autolysis: mos luminal epithelium sloughed. One cryptal PMN cast. Patchy, narrow lymphocytic band round muscularis mucosae.	**(2)** Mod eosins in LP.	**(2)** Mod eosins in LP	**(6)** Moderate autolytic epithelial sloughing in one section.	**(2)** Moderate autolysis. Possible bacteria in crypts. Few eosins in LP.	**(2)** Mod eosins in LP.	**(5)** Numerous slender, spiral, Gram neg., argyrophilic (Warthin-Starry) bacteria in crypts. EM: curved bacteria, not closely adherent
Spiral colon ('2')	**(2)** Luminal enterocytes ragged, no evidence of adherent bacteria	**(2)** Mild autolytic sloughing of epithelium. LP includes plasma c and scattered eosins.	**(3)** >60% epithelial loss. Poor section, no evidence of attached bacteria.	**(4)** NAD	**(2)** Few eosins in LP.	**(2)** Mod eosins in LP. Scattered necrosis in LP beneath epithelium.	**(4)** 1/2 sections moderately autolysed. Scattered eosins in LP.	**(2)** Possible Gram neg bacteria in crypts. Mod eosins in LP.	**(8)** Moderately autolysed, little luminal epithelium left.	**(2)** NAD
Rectum	No sample, fatty tissue only.	**(3)** Sparse population of lymphocytes in LP, compared with rest of intestine.	**(3)** Scattered single-cell necrosis in LP.	**(2)** A few eosins in LP. Gram stain NAD.	**(2)** NAD	**(2)** Focal epithelial necrosis and sloughing with associated PMN. Gram stain NAD.	**(2)** Scattered PMN and eosins in LP.	**(2)** Occasional eosins in LP.	**(2)** Moderate autolysis. Plasma cells in LP.	**(2)** NAD
Tonsil	Normal, active germinal centres.	1/2 present. Normal, active germinal centres.	No lymphoid tissue present.	Plant matter in crypts, with PMN exudate and infiltration of adjacent crypt epithelium. Focus of PMN and necrosis in pharyngeal epithelium. Not evident on Gram.	As controls.	1/2 tonsils present. One subepithelial focus of necrosis and mixed inflammatory cells. O157 ipx-lesion not seen.	Plant matter in crypts with surrounding PMN exudate.	Plant matter in crypts, with PMN. Lymphocytic - PMN epithelial infiltrate. Bacterial micro-abscess in sub-epithelial lymphoid tissue. Gram/ipx: not seen.	Plant matter in crypts with surrounding PMN exudate. PMN focus on edge of section. Neither discrete lesion nor bacteria evident on Gram.	1 focus of epithelial necrosis, with numerous embedded Gram neg bacteria. O157 ipx neg.
Mesenteric lymph nodes	Normal, active germinal centres .	Normal, active germinal centres; apoptosis within follicles and histiocytes within medullary sinuses.	Normal, active germinal centres. Some pigment in macrophages in distal MLN - probably haemosiderin.	Medullary sinuses of more cranial nodes are full of histiocytes, plus cells resembling haemosidero-phages.	As controls.	As controls	As controls	Small amounts of brown pigment in paracortex - may be in macrophages.	As controls	As controls

Notes: see page 178

Table 6-A continued

Lambs:	Uninoculated controls		1 day p.i.		3 days p.i.		7 days p.i.		15 days p.i.	
	9	10	11	12	13	14	15	16	17	18
Liver	NAD	Mild lympho-cytic portal infiltrate, with occasional eosins. Scattered, small foci of mononuclear inflammatory cells in parenchyma.	Small, sparse, multifocal, non-zonal mononuclear inflammatory foci in parenchyma.	Sinus spaces are wide - hepatocytes may be shrunken, possibly artefact.	NAD	Mild to moderate lymphocytic portal infiltrate. Occasional mononuclear cell focus in parenchyma.	NAD	Mild lymphocytic portal infiltrate and scattered parenchymal lymphoid foci.	Moderate lympho-plasmacytic portal infiltrate, with occasional mononuclear inflammatory foci in parenchyma.	Mild lymphocytic portal infiltrate, plus occasional parenchymal lymphocytic foci.
Kidney	NAD	NAD	NAD	NAD	Pale, eosinophilic, granular material in Bowman's space of many glomeruli.	A little eosinophilic material in some Bowman's spaces.	NAD	NAD	NAD	A little eosinophilic material in some Bowman's spaces, also in tubule lumina.
Spleen	Congested. Active lymphoid follicles	Congested. Active lymphoid follicles. Some leucocyte aggregates, including many PMN, in red pulp.	NAD	Moderate siderosis, congested.	NAD	NAD	NAD	NAD	Active lymphoid follicles.	Congested, active lymphoid follicles.
Other					Submandibular MLN: Normal, established GC activity.	Submandibular MLN: NAD				Pancreas and lymph nodes NAD.

Notes:
Shaded samples were inadequately preserved by the criteria in Section 6.3.1.
Intestinal mucosal preservation was good unless stated otherwise.
'Mod eosins in LP' indicates approximately evenly distributed eosinophils unless stated otherwise.
AE: attaching-effacing. **Eosin(s)**: eosinophil(s). **EP/GP**: excellent/good preservation.
Glob l'cytes: globular leucocytes. **ipx**: immunoperoxidase stain. **Low / mod**: low / moderate (density).
LP: lamina propria **LPC**: lamina propria cellularity. **PMN**: polymorphonuclear neutrophils.
NAD: no abnormalities detected. **V:Cr**: villus:crypt ratio.
Green text highlights deviations from excellent tissue preservation. Red text highlights histological features of potential interest.
Olive text highlights ipx findings. Blue text highlights electron microscopic findings.

Table 6-B: Histopathological findings in Experiment 6/2 (four-strain STEC O157:H7 inoculum in six-week old lambs) *Continued on page 180*

Lambs:	8 days p.i. 19	14 days p.i. 20	21 days p.i. 21	28 days p.i. 22	Uninoculated controls 27	 28
Rumen	**(2)** EP. Heavy surface colonisation (keratinised layers).	**(2)** EP. NAD	**(2)** EP. NAD	**(2)** EP. NAD	**(2)** EP. Occasional inflammatory focus in epithelium	**(2)** EP. Occasional PMN clusters in superficial epithelium
Duodenum	**(2)** GP· epithelial lifting. V:Cr 1:1. Mod LPC: mod, diffuse eosins, and plasma c. Low, mixed epithelial leucocytes. Local acute villitis. Adherent surface bacteria ++. Ipx. NAD	**(2)** EP. V:Cr norm. Mod LPC: LP oedematous, low, diffuse eosins. Minimal epithelial leucocytes. 1 small patch of bacteria on surface. Ipx: no adherent surface bacteria.	**(2)** EP. V:Cr normal. Mod LPC: mod plasma c, low eosins. Minimal epithelial leucocytes. Adherent surface debris +	**(2)** EP. V:Cr normal. Mod LPC: mod plasma c, low eosins. Minimal epithelial leucocytes. Adherent surface debris +. Prominent lacteals.	**(2)** EP. V.Cr 1-2:1 Mod LPC: plasma c and scattered eosins. Low epithelial leucocytes.	**(2)** EP. V:Cr 1-2:1 Mod LPC: plasma c and scattered. PMN and eosins. Low-mod mononuclear epithelial leucocytes. Adherent surface debris.
Jejunum	**(2)** EP. V:Cr norm. Mod LPC: mod eosins. Low mononuclear epithelial leucocytes. Adherent surface debris ++.	**(2)** EP. V:Cr normal. Mod LPC: LP mild oedema, mod, diffuse eosins. Low mononuclear cell epithelial leucocytes.	**(2)** GP. V:Cr normal. Mod LPC: mod, diffuse eosins, low plasma c. Low-mod mononuclear cell epithelial leucocytes. Adherent surface bacteria ++. Ipx: not O157	**(2)** EP. V:Cr 2-3:1 Mod LPC: mod eosins, low glob l'cytes. Low epithelial leucocytes. Adherent surface bacteria +/- Prominent lacteals.	**(2)** EP. V:Cr c. 2:1 Mod LPC-scattered plasma c and low eosins. *Globidium* parasite present.	**(2)** EP. V:Cr 2-3:1 Mod LPC: low eosins. Mod epithelial leucocytes - mononuclear cell. Scattered cryptosporidia.
Ileum	**(6)** EP. V:Cr norm. Mod LPC: mod eosins. Low epithelial leucocytes. Scattered Peyer's patch apoptosis. Adherent surface debris +.	**(6)** EP. V:Cr normal. Mod LPC: mod, diffuse eosins. Minimal epithelial leucocytes. Scattered Peyer's patch apoptosis.	**(6)** EP. V.Cr normal. Mod LPC: mod, diffuse eosins. Minimal epithelial leucocytes.	**(6)** EP. V:Cr 2-3:1 Mod LPC: mod eosins, low glob l'cytes. Low epithelial leucocytes. Adherent surface debris ++.	**(2)** EP. V:Cr c. 1:1 Mod LPC-low-mod eosins. Low epithelial leucocytes. Adherent surface debris.	**(2)** EP. V:Cr 1-2:1 Low-mod LPC: mod eosins. Low-mod epithelial leucocytes - mononuclear cell.
Caecum	**(6)** EP. Mod LPC: low-mod eosins and plasma c. Subepithelial multifocal PMN. Low epithelial leucocytes - PMN. Adherent surface debris +. Ipx: no evidence of lesions, non-specific brown debris ++	**(6)** GP. Mod LPC: mod eosins, plasma c and glob l'cytes. Minimal epithelial leucocytes. Sloughing epithelial cells + in lumen - pyknotic and markedly eosinophilic	**(6)** GP: sloughing epithelial cells but continuous cover. Mod LPC: mod plasma c, low eosin and glob l'cytes, occasional subepithelial PMN foci. Minimal epithelial leucocytes. Adherent surface debris.	**(6)** GP Sloughing epithelial cells are pyknotic. Mod LPC: mod plasma c, low eosins, glob l'cytes. Minimal epithelial leucocytes. Adherent surface debris +.	**(2)** EP. Mod LPC: scattered eosins, PMN and plasma c. Mod epithelial leucocytes - PMN.	**(2)** GP. Mod LPC: low eosins and plasma c. Low epithelial leucocytes. Spiral bacteria.
Proximal loop of colon ("1")	**(6)** GP Mod LPC: low-mod eosins and subepithelial multifocal PMN. Low epithelial leucocytes. Luminal PMN and epithelial cells +, adherent surface debris +	**(6)** GP: short stretch of sloughing Mod LPC: low eosins, plasma c and glob l'cytes. Minimal epithelial leucocytes. Sloughing cells, as caecum Adherent surface debris ++. Ipx: NAD.	**(6)** EP. Mod LPC: mod plasma c, eosins, glob l'cytes. Minimal epithelial leucocytes. Pyknotic sloughing epithelial cells. Adherent surface debris ++.	**(6)** EP. Mod LPC: mod plasma c, low eosins, glob l'cytes. Minimal epithelial leucocytes.	**(2)** GP (slough). Mod LPC: eosins low. PMN mod in subepithelial zone. Mod epithelial leucocytes -PMN. Spiral bacteria.	**(2)** EP. Mod LPC: mod plasma c, low eosins and scattered subepithelial PMN. Low epithelial leucocytes. Occasional coccidia

Notes: see page 185.

Table 6-B continued

Lambs:	8 days p.i. 19	14 days p.i. 20	21 days p.i. 21	28 days p.i. 22	Uninoculated controls 27	 28
Spiral colon ("2')	**(6)** GP. Mod LPC: low-mod eosins and subepithelial multifocal PMN. Low epithelial leucocytes. Luminal PMN and epithelial cells +, adherent surface debris +	**(6)** EP. Low-mod LPC: v. low eosins. Minimal epithelial leucocytes. Adherent surface debris +/-	**(6)** EP. Low/ mod LPC: plasma c, eosins, glob l'cytes. Minimal epithelial leucocytes. Irregular epithelial surface.	**(6)** EP. Mod LPC: mod plasma c, a few eosin and glob l'cytes. Adherent surface debris +++. Minimal epithelial leucocytes.	**(2)** EP. LPC: mod - plasma c and scattered eosins. Low epithelial leucocytes. Adherent surface debris and bacteria +++.	**(2)** EP. Low LPC: mod plasma c and scattered eosins. Frequent subepithelial necrotic foci with PMN. Low epithelial leucocytes.
Terminal colon ("3')	**(6)** EP. Low LPC: low eosins and multifocal subepithelial PMN with necrotic debris. Epithelial surface irregular.	**(7)** EP. Low LPC. Minimal epithelial leucocytes. Adherent surface debris +/-	**(7)** EP. Low LPC: plasma c. Minimal epithelial leucocytes. Adherent surface bacteria +.	**(6)** EP. Low LPC: low plasma c. Minimal epithelial leucocytes.	**(2)** EP. LPC: low - scattered plasma c and frequent subepithelial PMN with necrotic debris. Epithelial leucocytes - cluster PMN	**(2)** EP. Low LPC- scattered plasma c. Frequent subepithelial necrotic foci with PMN. Low epithelial leucocytes.
Rectum	Not sampled	Not sampled	Not sampled	Not sampled	**(2)** EP. LPC: low - scattered PMN, esp. subepithelial.	**(2)** EP. Low LPC: low plasma c. Occasional subepithelial necrotic foci with PMN. Low epithelial leucocytes. Epithelium corrugated and rough locally.
Mes. lymph nodes	Diffuse and focal eosins and diffuse macrophages in medulla.	A few medullary eosins and surgical haemorrhage.	A few medullary eosins.	A few medullary eosins.	Scattered PMN.	Perinodal parasite *Globidium*? in 1/3 nodes.
Tonsil	Cryptitis associated with plant matter. Bacteria in pharyngeal and crypt epithelium with PMN. Ipx: crypt bacteria seen, not staining	Cryptitis and associated epithelial PMN and bacteria. Ipx: Bacteria not specifically staining	Cryptitis. Focal crypt epithelium necrosis with rod bacteria, also bacteria in crypts.	NAD	Cryptitis- adventitious plant material, bacteria and PMN.	Cryptitis, including bacteria and epithelial PMN.
Liver, Kidney Spleen	Liver: mild lympho-eosinophilic portal infiltrate, scattered mixed inflammatory foci in parenchyma. Kidney: amorphous eosinophilic material in some distal convoluted tubules and collecting ducts.	Liver: very mild lympho-eosinophilic portal infiltrate, plus tiny sinusoid PMN foci.	Liver: mild, mixed portal infiltrate, tiny sinusoid PMN foci. Kidney: very mild, mixed renal corticomedullary inflammatory infiltrates.	Liver: very mild lympho-eosinophilic portal infiltrate. Kidney: Scattered mineralisation of renal medullary tubules.	Lymphocytic portal infiltrate.	Light, mixed portal infiltrate.

Notes: see page 185.

Table 6-C: Histopathological findings in Experiment 6/3 (single-strain STEC O157:H7 inoculum in six-week old lambs) *Continued on page 182*

Lambs	6 days p.i.	17 days p.i.	18 days p.i.	19 days p.i.	Uninoculated controls (also in Table 6-B)	
	23	24	25	26	27	28
Rumen	**(2)** EP. NAD	**(2)** EP. NAD	**(2)** EP. NAD	**(2)** EP. 1 small inflammatory focus in epithelium, with bacteria, not on ipx slide.	**(2)** EP. Occasional inflammatory focus in epithelium	**(2)** EP. Occasional PMN clusters in superficial epithelium
Duodenum	**(3)** GP, villus tip autolysis locally. V:Cr 1:1, flattened. Mod LPC, mod plasma c, eosins, low PMN. Low epithelial leucocytes - PMN.	**(2)** GP, some villus tip autolysis. V:Cr 1:1, slender. Mod LPC, mod eosins and plasma c. Low epithelial leucocytes - l'cytes. Adherent surface debris +.	**(2)** EP. V:Cr 1:1, norm morphology. Mod LPC, mod plasma c, low eosins. Low epithelial leucocytes. Focal coccidia.	**(2)** GP, some autolysis of villus tips. V.Cr 1:1, normal morphology. Low LPC, plasma c, low eosins. Low epithelial leucocytes - mononuclear cells.	**(2)** EP. V.Cr 1-2:1 Mod LPC - plasma c and scattered. eosins. Low epithelial leucocytes.	**(2)** EP. V:Cr 1-2:1 LPC mod - plasma c and scattered. PMN and eosins. Low-mod mononuclear epithelial leucocytes Adherent surface debris.
Jejunum	**(3)** EP. V:Cr 1:1 Mod LPC, mod eosins and plasma c. Mod epithelial leucocytes - l'cytes.	**(2)** GP, some epithelial lifting. V:Cr 1:1, Peyer's patches - more ileal than jejunal. Mod LPC, mod-high eosins. Coccidia common. Low epithelial leucocytes.	**(2)** GP: epithelial lifting. V:Cr 2:1. Mod-high LPC, mod eosins, low plasma c. Low epithelial leucocytes. Adherent surface debris ++. Parasite in crypts	**(2)** GP, some autolysis of villus tips. V.Cr 2:1. Mod LPC, mod eosins, low plasma c. Low epithelial leucocytes. Coccidia.	**(2)** EP. V:Cr c. 2:1 Mod LPC-scattered plasma c and low eosins. *Globidium* parasite present.	**(2)** EP. V:Cr 2-3:1 Mod LPC - low eosins. Mod epithelial leucocytes - mononuclear cell. Scattered cryptosporidia.
Ileum	**(6)** EP. Villi short (V:Cr 1:1). Mod LPC, mod-marked eosins, plasma c, plus coccidial cysts. Inflammatory debris frequent in crypts. Epithelium often contains coccidia. Minimal epithelial leucocytes.	**(6)** GP, some epithelial lifting. V:Cr 1 - 1.5:1 villi broad and stumpy. High LPC, mod eosins. Mod epithelial leucocytes: l'cytes and local PMN. Coccidia ++. Cryptal inflammatory debris. Local adherent bacteria, ipx: not O157	**(6)** GP, epithelial lifting and villus tip a"lysis. V:Cr 1-2:1. Mod LPC, mod eosins. Low, mononuclear epithelial leucocytes. Few epithelial coccidia.	**(6)** EP. V:Cr 1.5 - 2:1 Mod LP cellularity, mod eosins, low plasma c. Mod epithelial l'cytes. Occasional coccidia, inflammatory debris in crypts.	**(2)** EP. V:Cr c. 1:1 Mod LPC-low-mod eosins. Low epithelial leucocytes. Adherent surface debris.	**(2)** EP. V:Cr 1-2:1 Low-mod LPC - mod eosins. Low-mod epithelial leucocytes - mononuclear cell
Caecum	**(6)** EP. Mod LPC, mod eosins and plasma c, scattered PMN. Low epithelial leucocytes - PMN. Surface nematodes. Adherent surface debris ++.	**(6)** Poor Preservation, locally >>50% slough. Mod LPC, mod plasma c, low eosins. Low epithelial leucocytes.	**(6)** EP mod LPC, mod eosins and plasma c. Mod epithelial leucocytes - PMN and occasional crypt abscess. Focal PMN exudation with necrosis. AE lesions common, often mod extensive. Ipx: O115 positive. Possible spiral bacteria present.	**(6)** GP(A), Mod P (B). Individual cell sloughing. Mod-low LPC, mod plasma c, low eosins, globular leucocytes, occasional PMN. Parasites: encysted nematodes and probably coccidia. Probable spiral bacteria masses. Occasional crypt abscess.	**(2)** EP. Mod LPC - scattered eosins, PMN and plasma c. Mod epithelial leucocytes - PMN	**(2)** GP. Mod LPC - low eosins and plasma c. Low epithelial leucocytes. Spiral bacteria

Notes: see page 185.

Table 6-C continued

Lambs	6 days p.i. 23	17 days p.i. 24	18 days p.i. 25	19 days p.i. 26	Uninoculated controls (also in Table 6-B) 27	 28
Proximal loop of colon ("1")	(6) EP. Mod LPC, low-mod eosins, scattered plas c and PMN. Minimal epithelial leucocytes. Occasional inflammatory debris in crypt. Adherent surface debris ++.	(6) Poor preservation: c.40% slough overall. Mod LPC, med-low eosins and plasma c, occasional PMN. Low epithelial leucocytes.	(6) GP. localised sloughing. Mod LPC, low eosin, plasma c. Mod epithelial PMN. AE lesions small and mod PMN and pyknotic enterocytes common in lumen.	(6) Mod preservation: 80-90% cover. Mod LPC, mod plasma c, low eosins and lymphocytic basal aggregates. Necrotic PMN in subepithelial LP. Localised PMN in LP and epithelium.	(2) GP. (slough) Mod LPC - eosins low. PMN mod in subepithelial zone. Mod epithelial PMN. Spiral bacteria.	(2) EP. mod LPC - mod plasma c, low eosins and scattered subepithelial PMN. Low epithelial leucocytes. Occasional coccidia
Spiral colon ("2")	(7) EP. Mod LPC, low-mod eosins, plasma c, globular leucocytes. Low epithelial PMN.	(6) GP. Mod LPC, low eosins, plasma c, mast c. Minimal epithelial leucocytes. Adherent surface debris +.	(6) EP. Mod LPC, mod plasma c, low eosins. Low epithelial leucocytes. No AE lesions.	(6) EP. Low-mod LPC, mod plasma c. Low epithelial leucocytes.	(2) EP. LPC mod - plasma c and scattered eosins. Low epithelial leucocytes. Adherent surface debris and bacteria +++.	(2) EP. low LPC -mod plasma c and scattered eosins. Frequent subepithelial necrotic foci with PMN. Low epithelial leucocytes.
Terminal colon ("3")	(6) GP, epithelial cover complete, except focus of slough. of pyknotic epithelium. Mod LPC, low eosins, scattered plasma c. Minimal epithelial leucocytes.	(5) EP. Low-mod LPC, mod plasma c, low eosins. Low epithelial leucocytes. Adherent surface debris +.	(6) EP. Mod-low LPC, mod plasma c, low eosins. Low epithelial leucocytes. Epithelium irregular. No AE lesions.	(6) EP. Low LPC, Mod plasma c. Low epithelial leucocytes.	(2) EP. LPC low - scattered plasma c and frequent subepithelial PMN with necrotic debris. Epithelial clusters of PMN	(2) EP. Low LPC- scattered plasma c. Frequent subepithelial necrotic foci with PMN. Low epithelial leucocytes.
Rectum	(6) EP, Mod LPC, plasma c and few glob l, plus subepithelial PMN with focal necrosis. Epithelium focally ragged, no adherent surface bacteria.	(6) EP (A) and Mod P (80% present, B). Low LPC, Mod plasma c. Minimal epithelial leucocytes. Adherent surface debris +.	(6) GP, individual cell sloughing. Mod-low LPC, mod plasma c, low eosins, mod subepithelial PMN. Scattered AE lesions. Ipx: O115 positive. 1 crypt abscess.	(6) EP. Low LPC, mod plasma c, necrotic subepithelial PMN. Low epithelial leucocytes, some necrotic debris. Epithelium ragged locally.	(2) EP. LPC low - scattered PMN, esp. subepithelial.	(2) EP. Low LPC - low plasma c. Occasional subepithelial necrotic foci with PMN. Low epithelial leucocytes. Epithelium corrugated and rough locally.
Mes. lymph nodes	Eosinophils common.	Scattered PMN and eosins.	Scattered PMN	Macrophages in medullary sinuses. Parasite cell mass in one node - *Globidium*? not present on ipx slide	Scattered PMN	Perinodal parasite *Globidium*? in 1/3
Tonsil	Cryptitis, with large crypt bacterial mass.	Cryptitis, PMN infiltrating adjacent epithelial and lymphocytic tissue.	Cryptitis with accompanying bacteria	Cryptitis with bacteria and adventitious plant material. Ipx: not O157	Cryptitis- adventitious vegetation., bacteria and PMN	Cryptitis, including bacteria and epithelial PMN.
Liver Kidney Spleen	Mild lympho-plasmacytic portal infiltrate.	Mild lympho-histiocytic infiltrate.	Occasional tiny PMN clusters in portal tracts and liver parenchyma.	Mild-mod lympho-histiocytic portal infiltrate and siderosis. Focal biliary tract epithelium necrosis.	Lymphocytic portal infiltrate.	Light, mixed portal infiltrate.

Notes: see page 185.

Table 6-D: Histopathological findings in Experiment 6/4 (single-strain non Shiga toxin-producing *E. coli* O157:H7 inoculum in six-week old lambs)
Continued on page 184

Lambs	1 day p.i. 29	3 days p.i. 30	7 days p.i. 31	9 days p.i. 32	Uninoculated controls 33	 34
Rumen	(2) A few eosins in lamina propria.	(2) Many surface-adherent bacteria. 1 acute inflammatory focus on surface. O157 ipx: no evidence of attached O157.	(2) NAD	(2) NAD	(2) NAD	(2) NAD
Duodenum	(4) EP. V:Cr 1:1. Mod-high LPC: mod plasma c, low eosins and PMN, some siderophages. Mod, mixed epithelial leucocytes.	(4) EP. V:Cr 0.5-1:1, villi broad and stumpy. Mod LPC: mod-high plasma c, low eosins, mutifocally mod PMN. Minimal epithelial leucocytes, occasional foci of PMN.	(4) EP. V:Cr 1:1. Mod LPC: mod plasma c, scattered eosins and PMN. Low mononuclear cell epithelial leucocytes.	(4) EP. V:Cr 1.5:1. Mod LPC: mod plasma c, scattered eosins. Low, mononuclear cell epithelial leucocytes. Occasional crypt abscesses.	(4) EP. V:Cr 1:1. Mod LPC: mod plasma c, low eosins, patchy, low PMN. Low mononuclear cell epithelial leucocytes. Occasional inflammatory debris in crypts. 1 luminal nematode, 1 coccidial mass in epithelium.	(4) EP. V:Cr 1:1, villi typically as wide as tall. Mod LPC: mod plasma c, low eosins. Low mononuclear cell epithelial leucocytes.
Jejunum	(4) EP. V:Cr 1- 1.5:1. Mod LPC: mod eosins, occasional plasma c. Occasional crypts contain eosinophil debris. Coccidia common in epithelium.	(4) EP. V:Cr 1:1, villi wide. Mod LPC: mod eosins, scattered PMN and plasma c. Minimal epithelial leucocytes. Inflammatory debris in many crypts. Coccidia common in epithelium.	(4) GP - some epithelial loss at villus tips. V:Cr 1.5:1. Mod LPC: low eosins, scattered PMN and plasma c. Low mononuclear cell epithelial leucocytes.	(4) EP or poor preservation, correlates with lumen being opened or not before fixation. V:Cr 1.5:1. Mod LPC: mod eosins, low plasma c. Coccidia common in epithelium. Moderate mononuclear cell epithelial leucocytes. Scattered surface-adherent bacteria - not typical AE.	(4) EP. V:Cr 1:1. Mod LPC: mod-high eosins, mod plasma c and PMN. Minimal epithelial leucocytes. Inflammatory debris in many crypts, occasional microabscesses in lamina propria. Coccidia common in epithelium.	(4) GP - epithelium lifting at villus tips. V:Cr 1.5-2:1. Mod LPC: mod-high eosins, low plasma c, scattered PMN in subepithelial zone. Low mononuclear cell epithelial leucocytes. Inflammatory debris common in crypts. Coccidia common in epithelium.
Ileum	(6) EP. V:Cr 1.5:1. Mod LPC: mod eosins. Low PMN epithelial leucocytes, especially on follicle domes. One very small cluster of surface-adherent bacteria.	(6) EP. V:Cr 1.5:1. Mod LPC: mod eosins, low plasma c, patchy, low PMN. Minimal epithelial leucocytes. Eosinophil debris in many crypts. Coccidia common in epithelium.	(6) GP - minor epithelial lifting. Mod LPC: mod eosins, low plasma c, patchy PMN. Minimal epithelial leucocytes. Small crypt abscesses common. Coccidia common in epithelium.	(6) EP. V:Cr 1.5-2:1. Mod-high LPC: mod to focally high eosins; scattered plasma c, patchy, focally high PMN with luminal exudation and one associated luminal bacterial mass. Moderate, mixed epithelial leucocytes. Crypt abscesses common, coccidia present.	(2) EP. V:Cr 1-1.5:1. Mod LPC: mod eosins. Low mononuclear cell epithelial leucocytes. Occasional inflammatory debris in crypts and in lumen. Occasional coccidia in epithelium.	(3) EP. V:Cr 1-1.5:1. Mod LPC: mod to focally high eosins, low plasma c. Low mononuclear cell epithelial leucocytes. Coccidia common in epithelium.

Notes: see page 185.

Table 6-D continued

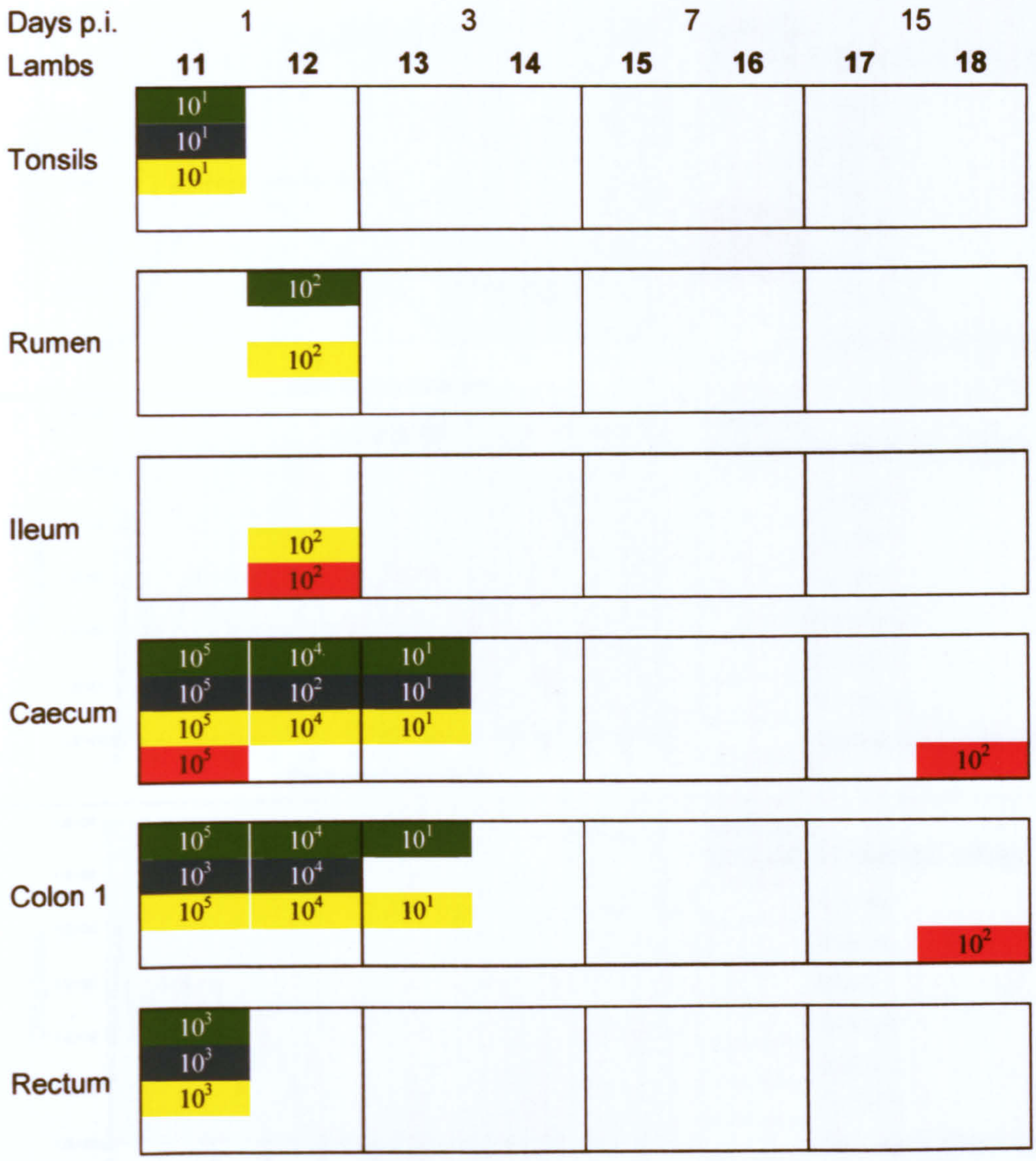
Lambs	1 day p.i. 29	3 days p.i. 30	7 days p.i. 31	9 days p.i. 32	Uninoculated controls 33	 34
Caecum	(6) EP. Mod LPC: mod plasma c, low eosins. Numerous, small AE-type lesions, covering 1 -10 enterocytes. Ipx O157 / O26: 2 x O26 positive lesions plus 1 x O157 positive lesion seen. Most (larger) lesions are O157 and O26 neg EM: AE lesions present in 'pop-off' sections.	(3) EP. Mod LPC: mod plasma c, low eosins, low subepithelial band of PNM. Coccidia common in epithelium. Surface-adherent bacteria present, but not typical of AE lesions.	(7) EP. Mod LPC: mod plasma c, low eosins and subepithelial band of PMN. Minimal epithelial leucocytes. 8 AE-type lesions, each covering 1-10 adjacent enterocytes. Ipx neg for O157 and O115 Occasional coccidia and 1 nematode in epithelium.	(6) EP. Mod LPC: mod plasma c, low eosins, multifocal PMN, including dense PMN exudate associated with bacterial mass in lamina propria. Scattered subepithelial necrotic debris. 2 small AE-type lesions, Ipx O157, O26 and O115: no AE lesions seen, bacterial mass neg.	(3) EP. Mod LPC: low eosins, scattered plasma c. Minimal epithelial leucocytes.	(3) EP. Mod LPC: mod plasma c, low eosins, scattered PMN in subepithelial zone. Minimal epithelial leucocytes.
Proximal loop of colon ('1')	(3) GP - occasional sloughing of short sections of epithelium. Mod LPC: mod eosins, low plasma c. Patchy mod-high PMN epithelial leucocytes. Many AE -type lesions covering 1-20 enterocytes each.	(5) EP. Mod LPC: low plasma c, eosins and PMN. Minimal epithelial leucocytes. 1 encysted nematode in epithelium.	(6) EP. Mod LPC: mod plasma c, low eosins, patchy, mod PMN in subepithelial zone. Minimal epithelial leucocytes. Many small or mod-sized AE-type lesions. Ipx neg for O157, O26 and O115 Occasional coccidia.	(6) GP - some lifting of epithelium. Mod LPC: low eosins, focus of PMN. Minimal epithelial leucocytes. 1 AE-type lesion, covering 6-7 epithelial cells. O157 and O26 ipx: no lesions seen.	(3) GP. Mod LPC: low eosins, mod plasma c, patchy PMN in subepithelial zone and migrating across epithelium. Many clusters of surface-adherent bacteria.	GP - much epithelial lifting, but little loss. Mod LPC: mod eosins and plasma c. Minimal epithelial leucocytes.
Spiral colon ('2')	(3) EP. Mod LPC: mod plasma c and eosins. Minimal epithelial leucocytes.	(6) EP. Low-mod LPC: low eosins, low, subepithelial band of PMN. Minimal epithelial leucocytes.	(6) EP. Mod LPC: scattered eosins, low plasma c, occasional PMN. Minimal epithelial leucocytes.	(6) EP. Mod LPC: mod plasma c, low eosins, patchy PMN. Minimal epithelial leucocytes. Occasional epithelial coccidia. 1 bacterial microabscess in lamina propria, not present on ipx.	(3) GP. Mod LPC: mod plasma c, low eosins. Coccidia common in epithelium.	(3) EP. Mod LPC: mod eosins and plasma c. Minimal epithelial leucocytes.
Terminal colon ('3')	(6) EP. Low LPC: mod plasma c, low-mod band of subepithelial PMN. Minimal epithelial leucocytes. 7 AE-type lesions. O157 / 026 ipx: lesions are all neg for both serogroups.	(6) GP- Lifting of epithelium but little loss. Low-mod LPC: low plasma c, scattered subepithelial PMN. Minimal epithelial leucocytes.	(6) EP. Low LPC: scattered eosins and plasma c, scattered PMN and necrotic debris in subepithelial zone. Minimal epithelial leucocytes.	(6) EP. low LPC: low plasma c, sparse PMN and necrotic debris in subepithelial zone. Minimal epithelial leucocytes.	(3) EP. Low LPC: mod plasma c, scattered PMN and necrotic debris in subepithelial zone. Minimal epithelial leucocytes.	(3) EP. Low LPC: occasional PMN, especially in subepithelial zone. Minimal epithelial leucocytes.
Rectum	(6) GP - lifting and sloughing of epithelium. Low LPC: low plasma c, sparse, subepithelial band of PMN. Numerous small AE-type lesions.	(6) GP - Lifting of epithelium but little loss. Low-mod LPC: mod plasma c. Much surface-adherent bacteria and debris, no AE-type lesions.	(6) EP. Low-mod LPC: low plasma c, scattered subepithelial PMN. Minimal epithelial leucocytes.	(4) EP. Low-mod LPC: mod plasma c, multifocal PMN, PMN and necrotic debris subepithelial band. Many small crypt abscesses, +/- bacteria. Few PMN in epithelium.	(3) EP. Low LPC: low plasma c. Minimal epithelial leucocytes. Patchy, mod PMN in subepithelial zone.	(3) EP Low-mod LPC: low plasma c, scattered subepithelial necrotic debris. Minimal epithelial leucocytes. Bacterial aggregates common in lumen and on surface.
Liver kidney Spleen	Liver: Sparse lymphocytic portal infiltrate.	NAD	Liver: Mild, mixed portal inflammatory infiltrate.	NAD	Liver: very mild lympho-plasmacytic portal infiltrate.	Liver: mild, mixed portal inflammatory infiltrate.

Notes: see page 185.

Notes for tables 6-B to 6-D:

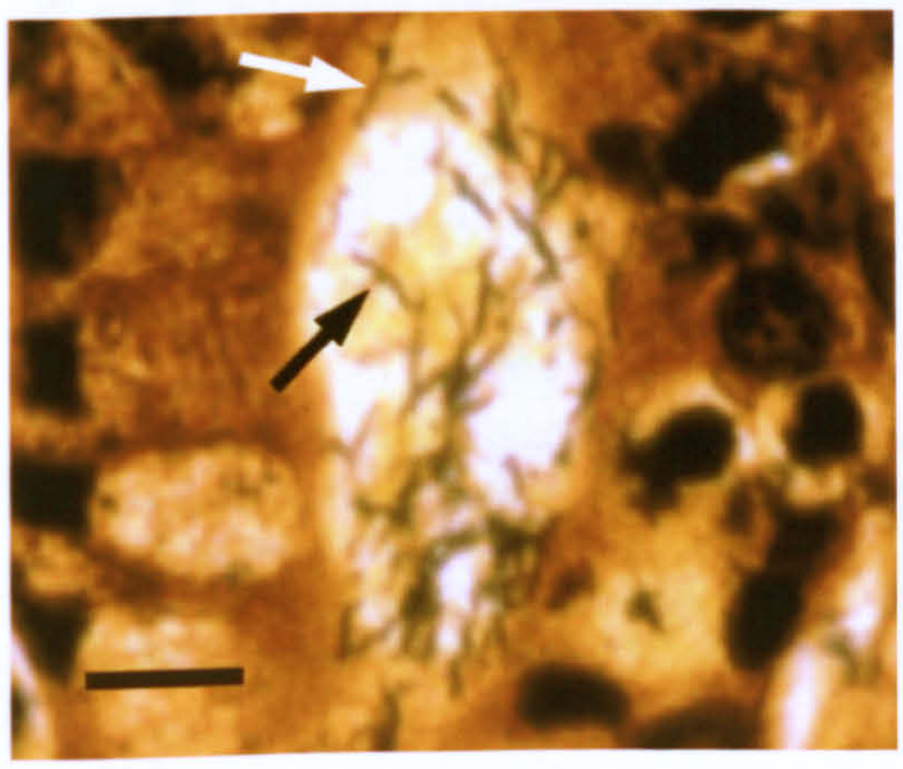
Villus morphology and lymphocyte populations in the lamina propria are normal unless stated otherwise.
AE: attaching-effacing.
Eosin(s): eosinophil(s).
EP/GP: Excellent/good preservation.
ipx: immunoperoxidase stain.
Low/mod: Low/moderate (density).
LPC: Lamina propria cellularity.
PMN: Polymorphonuclear neutrophils.
NAD: no abnormalities detected.
V:Cr: villus:crypt ratio.
Green text highlights deviations from excellent tissue preservation.
Red text highlights histological features of potential interest.
Olive text highlights ipx findings.
Blue text highlights electron microscopic findings.

Figure 6-a: Bacteriological findings from tissue samples in Experiment 6/1



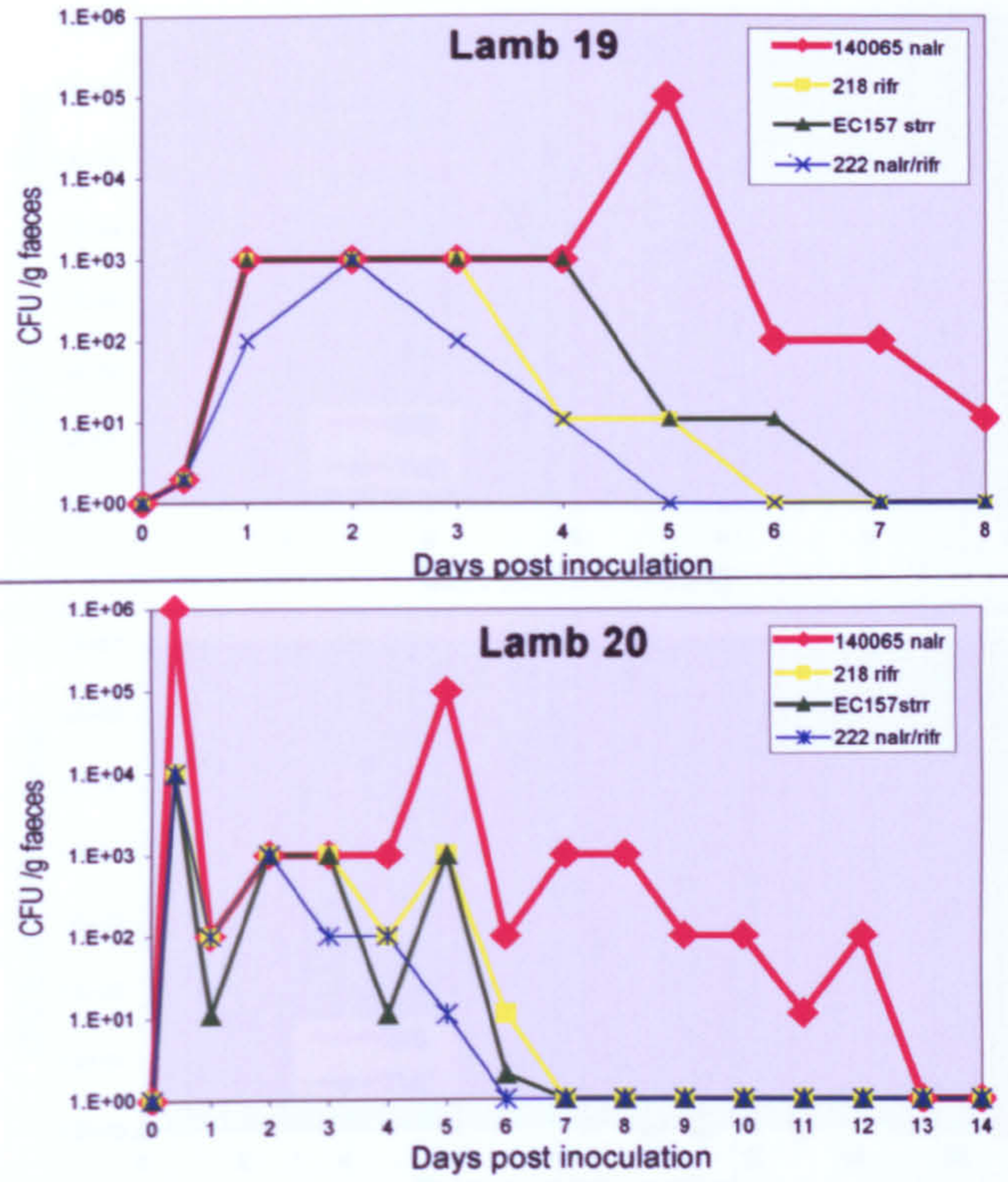
Numbers are estimates of the concentration of the particular experimental strain, in cfu/g tissue, made using the semiquantitative Most Probable Number technique described in Section 2.2.9.

Figure 6-b: Spiral bacteria in colon



Lamb 18, colon. Silver-stained spiral bacteria (examples arrowed) are present in a crypt lumen. Warthin-Starry, bar = 10 μm.

Figure 6-c: Bacteriological findings from faeces samples (left) and tissue plus intestinal contents samples (right) in Experiment 6/2



Lamb 19
Samples 8 days p.i. (cfu/g, strain)

	Tonsils
	Rumen
	Ileum
1, nal^r	Caecum
1, nal^r	Colon 2
	Colon 3
	Mesenteric lymph nodes

Lamb 20
Samples 14 days p.i. (cfu/g)

	Tonsils
	Rumen
	Ileum
	Caecum
	Colon 2
	Colon 3
	Mesenteric lymph nodes

Lamb 21
Samples 21 days p.i. (cfu/g)

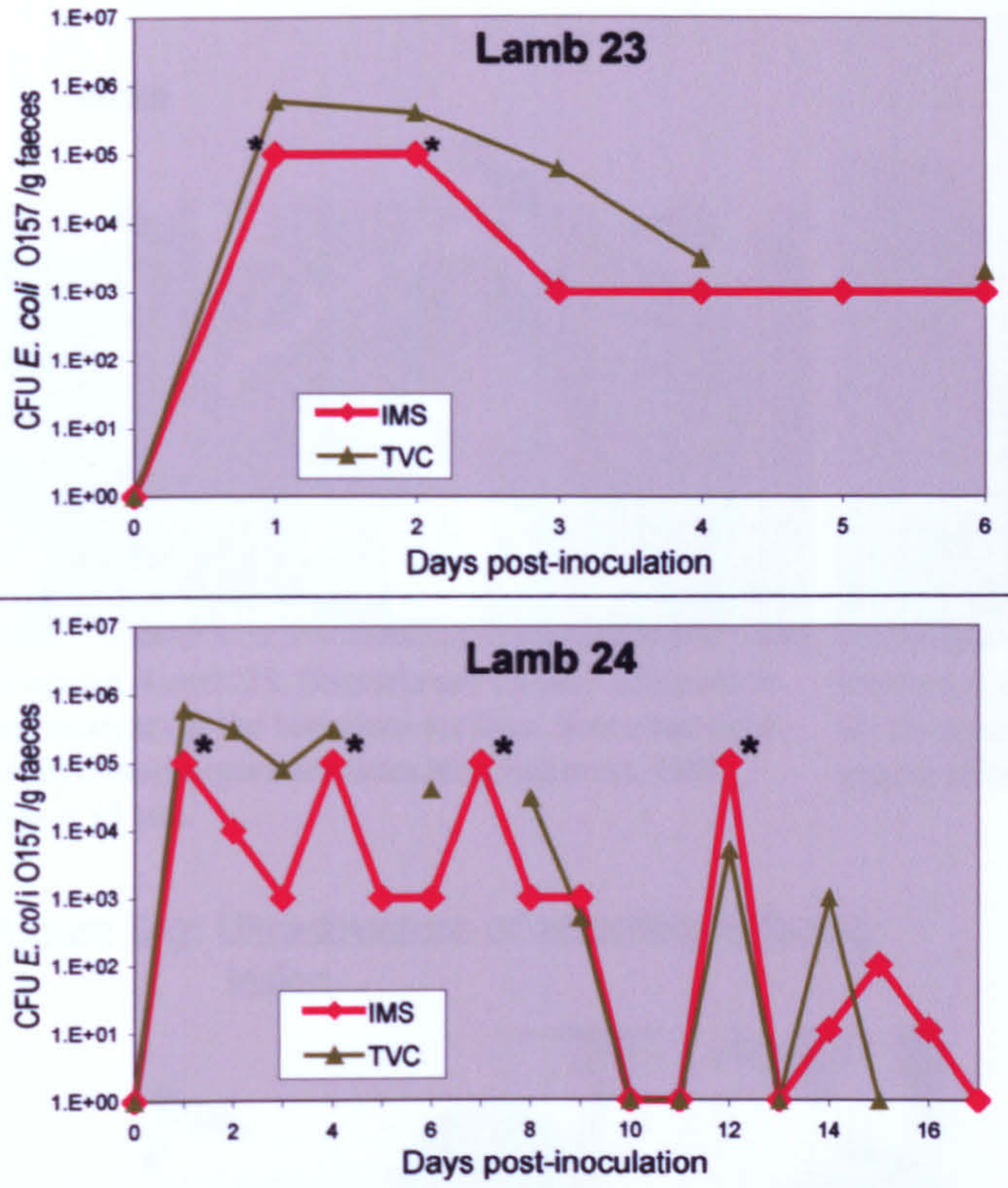
	Tonsils
	Rumen
	Ileum
	Caecum
	Colon 2
	Colon 3
	Mesenteric lymph nodes

Lamb 22
Samples 28 days p.i. (cfu/g)

	Tonsils
	Rumen
	Ileum
	Caecum
	Colon 2
	Colon 3
	Mesenteric lymph nodes

Notes: One (1) has been added to all excretion data points to allow a logarithmic plot of data which includes zero values. Detection was via a semiquantitative IMS technique. Many data points at and above 10^3 cfu/g represent the maximum dilutions tested for that sample, i.e. a higher value might have been obtained if more dilute samples had been tested. Asterisked points represent samples where a dilution series was not prepared and detection was from a neat sample only.

Figure 6-d: Bacteriological findings from faeces samples (left), and tissue and intestinal contents samples (right) in Experiment 6/3

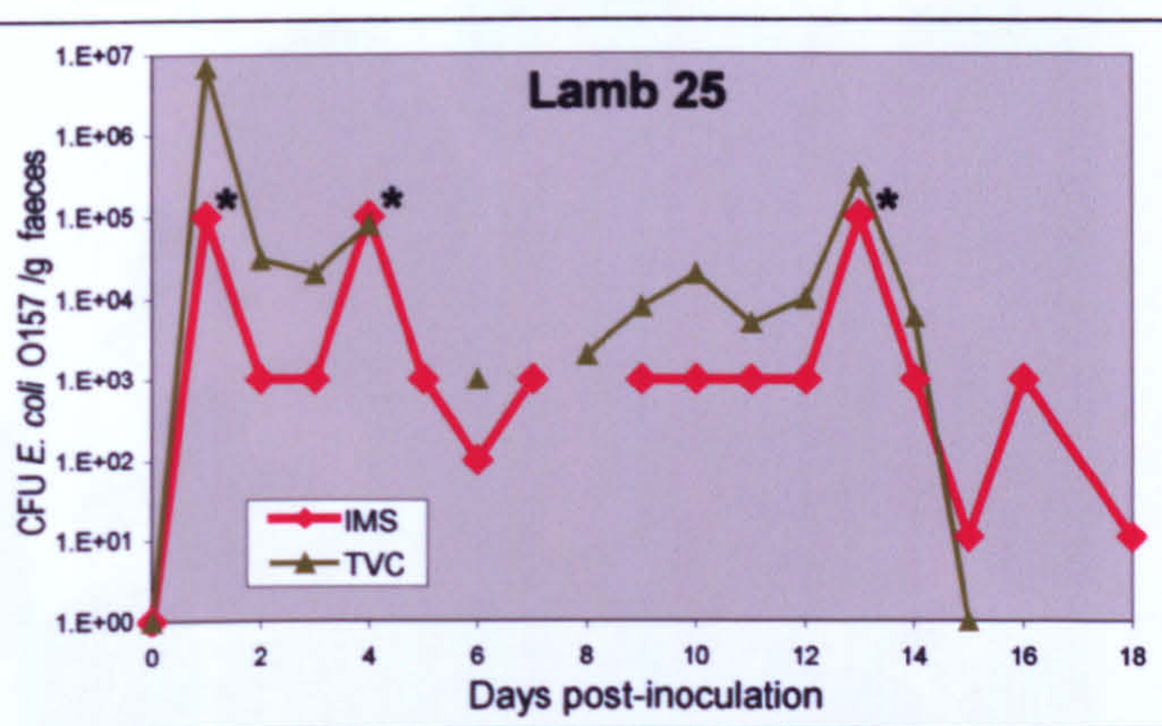


Lamb 23, 6 days p.i. (cfu/g)

	Tonsils
10^1	Rumen
10^1	Ileum
10^1	Caecum
$\geq 10^3$	Colon 2
10^1	Colon 3 tissue
10^3	Colon 3 contents
10^1	Rectum tissue
10^3	Rectum contents
	Mesenteric lymph nodes

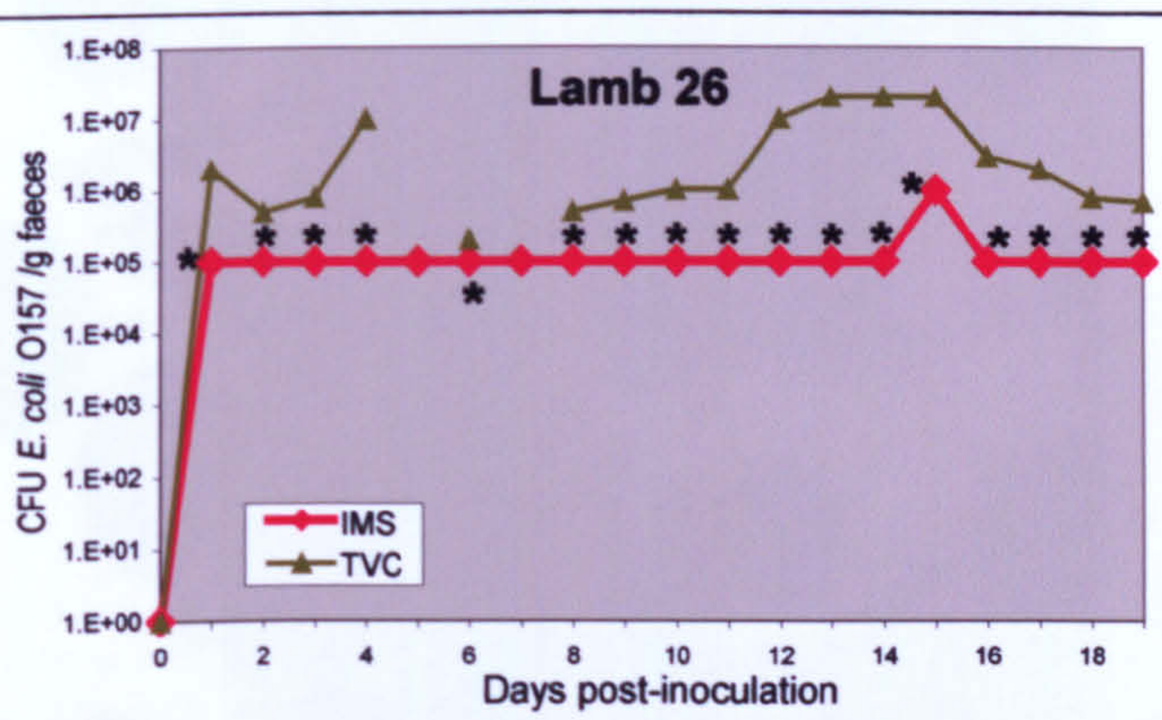
Lamb 24, 17 days p.i. (cfu/g)

	Tonsils
	Rumen
	Ileum
	Caecum
	Colon 2
	Colon 3 tissue
	Colon 3 contents
	Rectum tissue
	Rectum contents
	Mesenteric lymph nodes



Lamb 25, 18 days p.i. (cfu/g)

	Tonsils
	Rumen
	Ileum
#	Caecum †
ǂ(O115)	Colon 2
	Colon 3 tissue
$\geq 10^0$ (1	Colon 3 contents
	Rectum tissue †
ǂ(O115)	Rectum contents
	Mesenteric lymph nodes

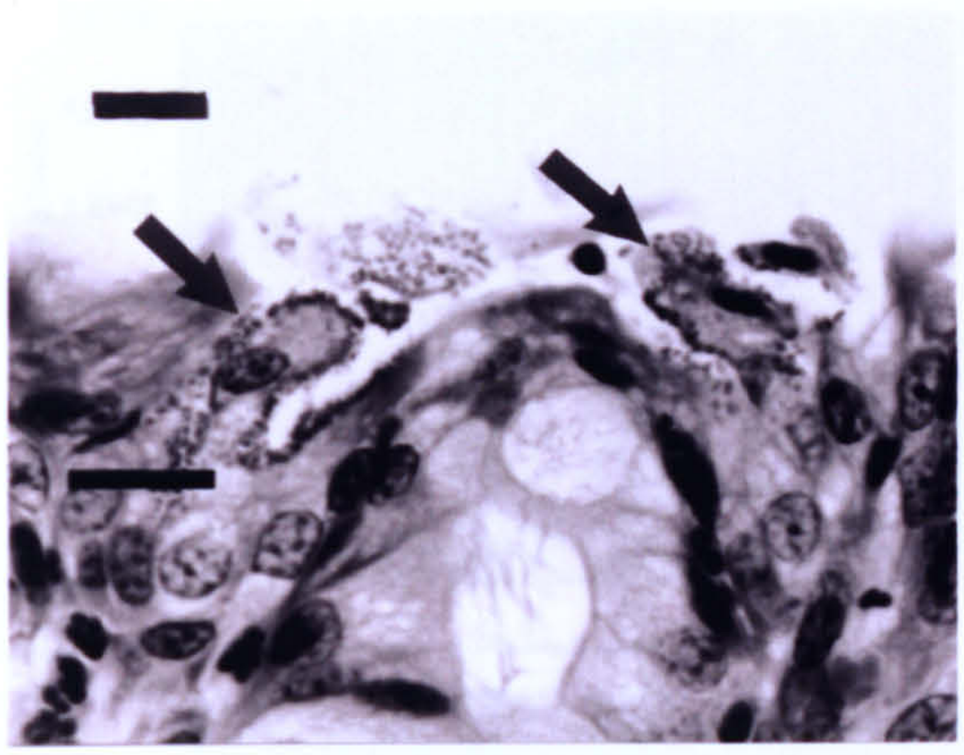


Lamb 26, 19 dpi (cfu/g)

	Tonsils
	Rumen
10^1	Ileum
$\geq 10^5$	Caecum
$\geq 10^5$	Colon 2
10^3	Colon 3 tissues
$\geq 10^5$	Colon 3 contents
10^3	Rectum tissues
$\geq 10^5$	Rectum contents
	Mesenteric lymph nodes

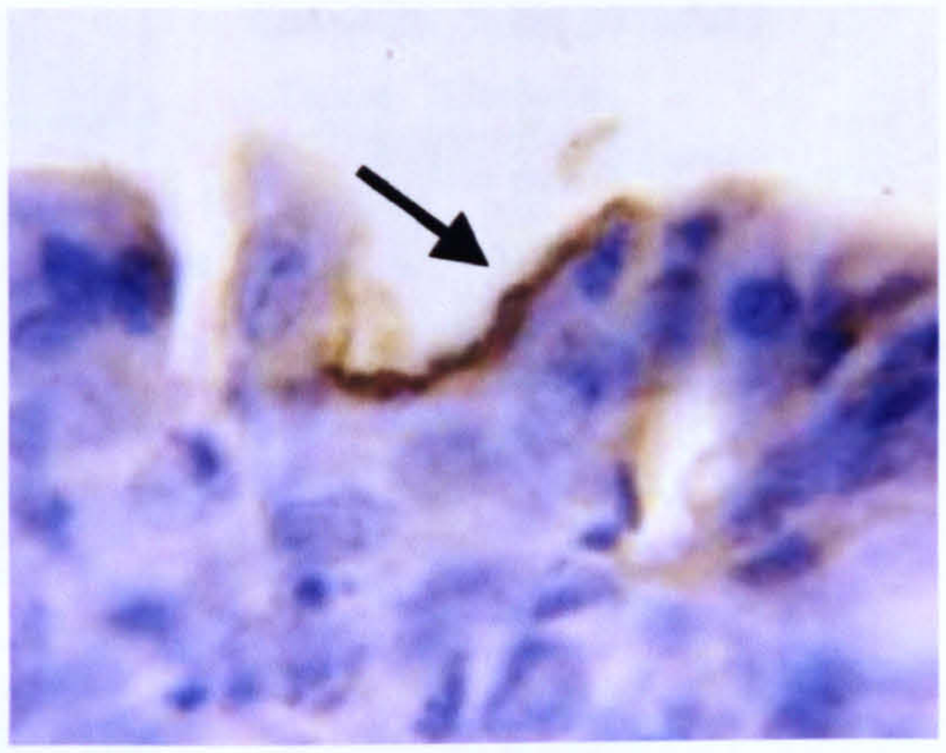
Notes: One (1) has been added to all excretion data points to allow a logarithmic plot of data which includes zero values. Faecal *E. coli* O157 were enumerated by semiquantitative IMS (red), and by direct culture of a dilution series (green), in parallel. Asterisked data points represent the maximum dilutions tested for that sample, i.e. a higher value might have been obtained if more dilute samples had been tested. (O115): *E. coli* O115:H- isolated. #: Results of pre-enriched direct subculture. † AE lesions seen.

Figure 6-e: Attaching-effacing lesion



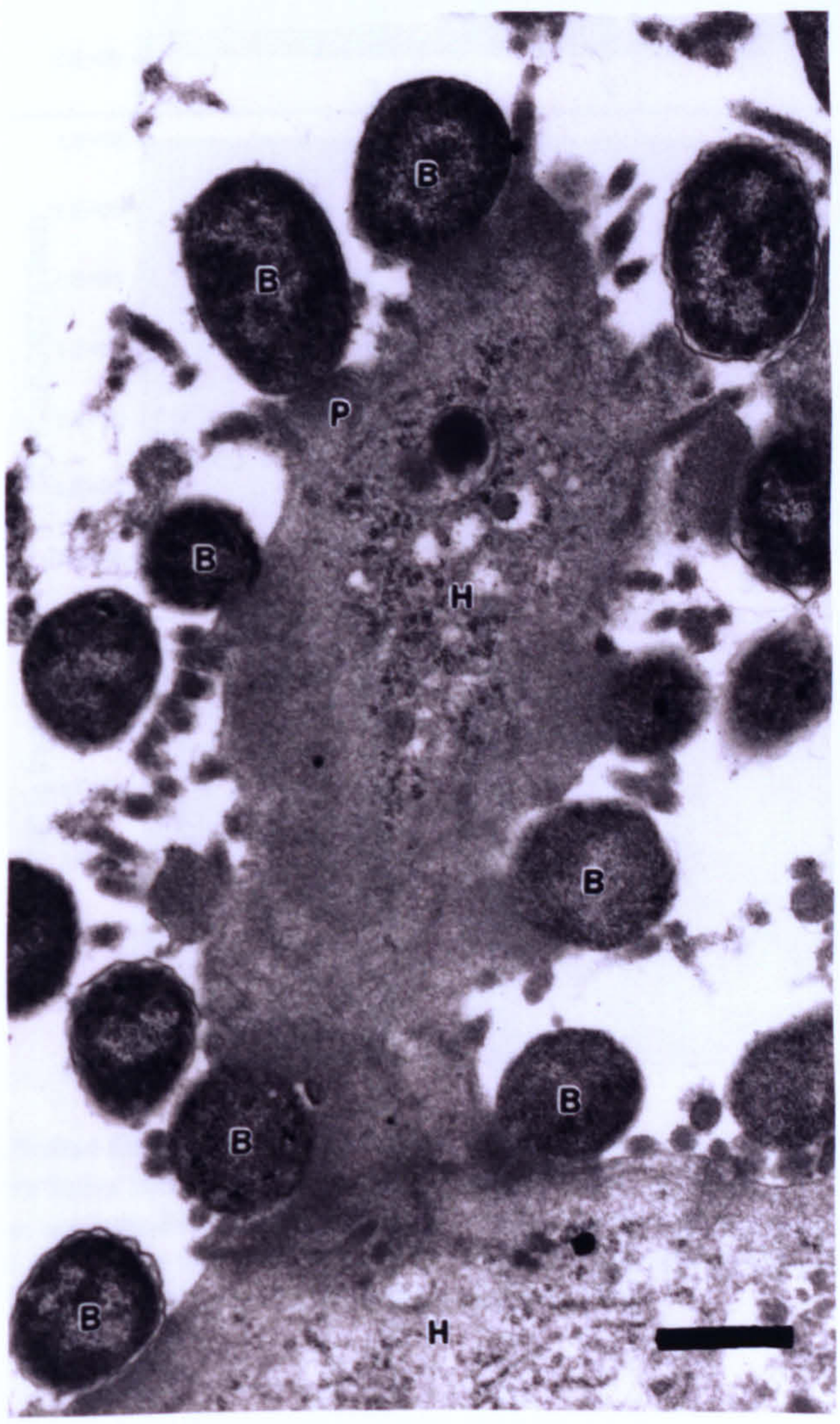
Caecum, Lamb 25. Bacteria are closely adherent to enterocytes at the intestinal surface. Some bacteria-coated enterocytes are detaching (arrows). H&E, bar = 15 μm.

Figure 6-f: Immunoperoxidase stained attaching-effacing lesion



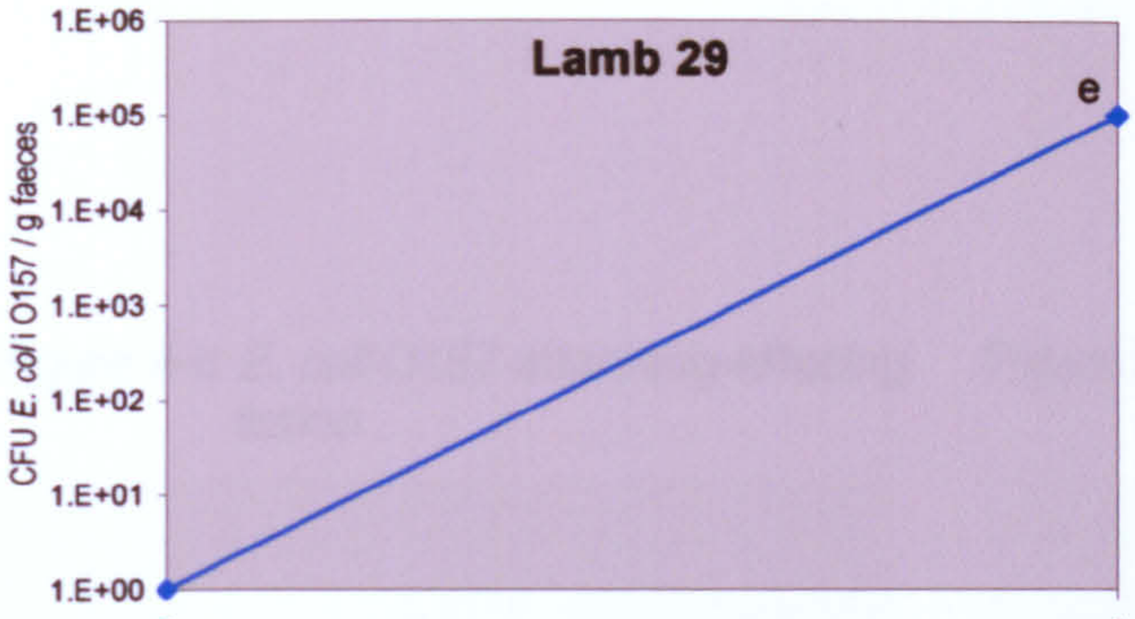
Rectum, Lamb 25. Immunolabelled mass of *E. coli* O115 organisms (arrow), adhering to the luminal aspect of enterocytes. O115 ipx, bar = 10 μm.

Figure 6-g: Ultrastructure of attaching-effacing lesion



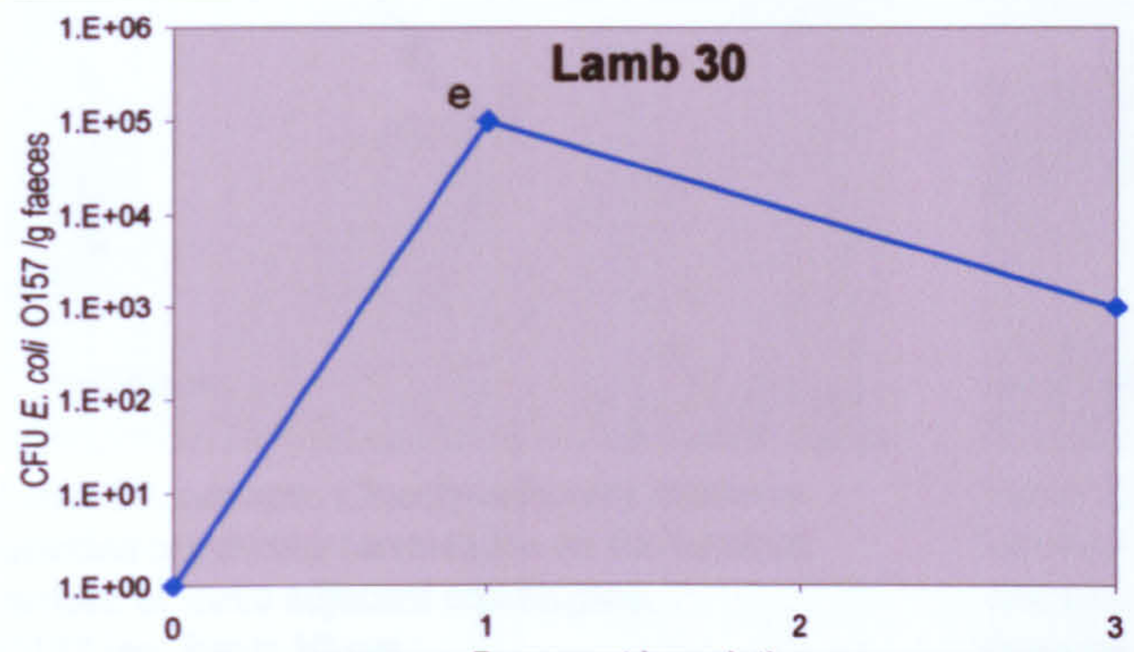
Caecum, Lamb 25. Host enterocytes (H) have lost their microvillous surface and bacteria (B) are intimately attached to the plasma membrane. Some pedestals (P) have formed. Bar = 750 nm

Figure 6-h: Bacteriological findings from faeces samples (left), and tissue and intestinal contents samples (right) in Experiment 6/4



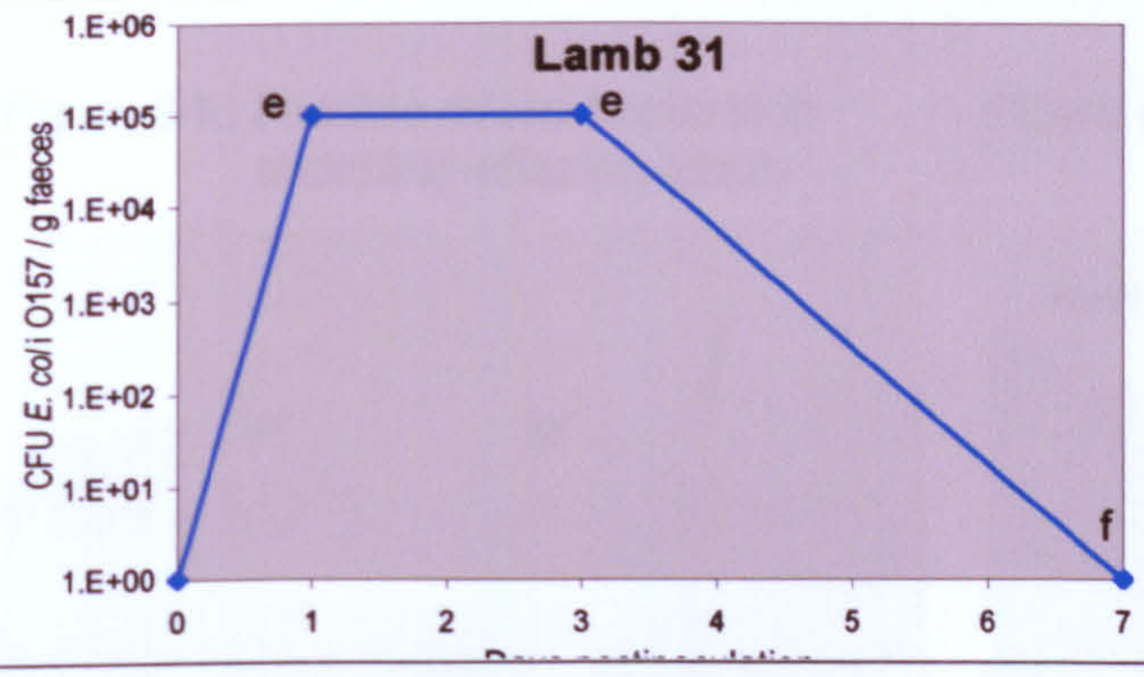
Lamb 29, 1 day p.i. (cfu/g)

Tissue	Contents	
≥10^{1} [a]	≥10^{1} [a]	Rumen
10^{0} (1)	ND	Duodenum
≥10^{1} [a]	≥10^{1} [a]	Jejunum
≥10^{1} [a]	≥10^{3} [b]	Ileum
10^{3}	≥10^{5} [c]	Caecum
≥10^{5} [c]	≥10^{5} [c]	Colon 1
≥10^{5} [c]	≥10^{5} [c]	Colon 3
≥10^{5} [c]	≥10^{3} [d]	Rectum



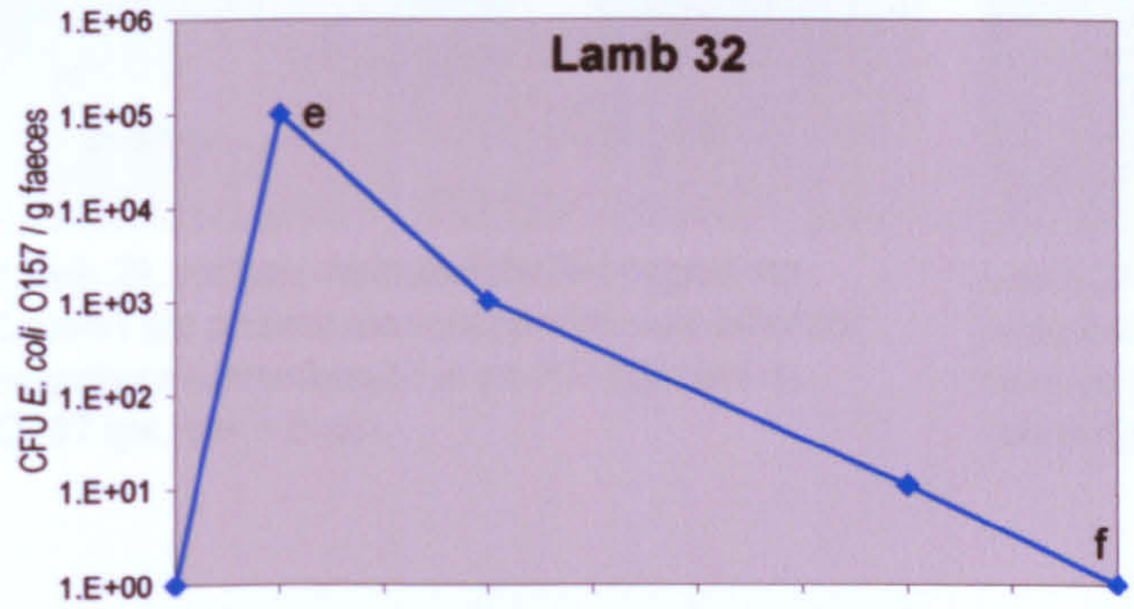
Lamb 30, 3 days p.i. (cfu/g)

Tissue	Contents	
10^{3}	10^{1}	Rumen
	ND	Duodenum
	10^{0} (1)	Jejunum
	ND	Ileum
10^{3}	10^{3}	Caecum
≥10^{5}	≥10^{5} [c]	Colon 1
10^{3}	10^{3}	Colon 3
10^{3}	10^{3}	Rectum



Lamb 31, 7 days p.i. (cfu/g)

Tissue	Contents	
	10^{1}	Rumen
		Duodenum
		Jejunum
		Ileum
	10^{1}	Caecum
		Colon 1
	ND	Colon 3
	10^{1}	Rectum

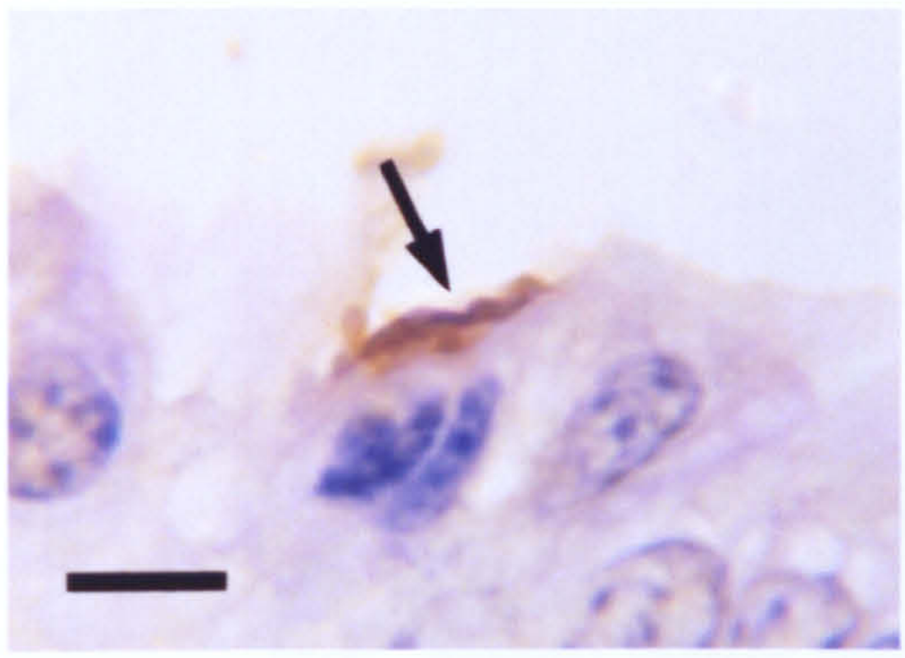


Lamb 32, 9 days p.i. (cfu/g)

Tissue	Contents	
10^{0} (1)	10^{1}	Rumen
	ND	Duodenum
		Jejunum
	ND	Ileum
f	f	Caecum
f	f	Colon 1
f	f	Colon 3
f	f	Rectum

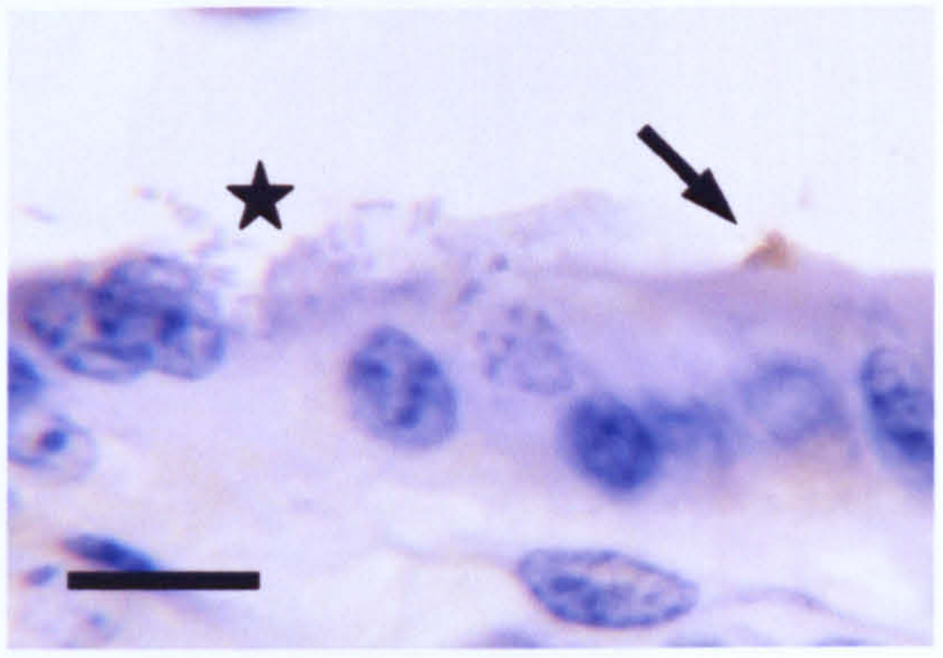
Notes: One (1) has been added to all excretion data points to allow a logarithmic plot of data which includes zero values. ND: not done (inadequate sample). a,b: Dilutions beyond 10^{1}(a) or 10^{3}(b) not done. c: uncountable at 10^{5} dilution. d: 10^{3} dilution results unclear. e: data points are maximum dilutions tested, i.e. a higher value might have been obtained if more dilute samples had been tested. f: Overgrowth by intestinal flora may have obscured *E. coli* O157

Figure 6-i: *E. coli* O157 attaching-effacing lesion



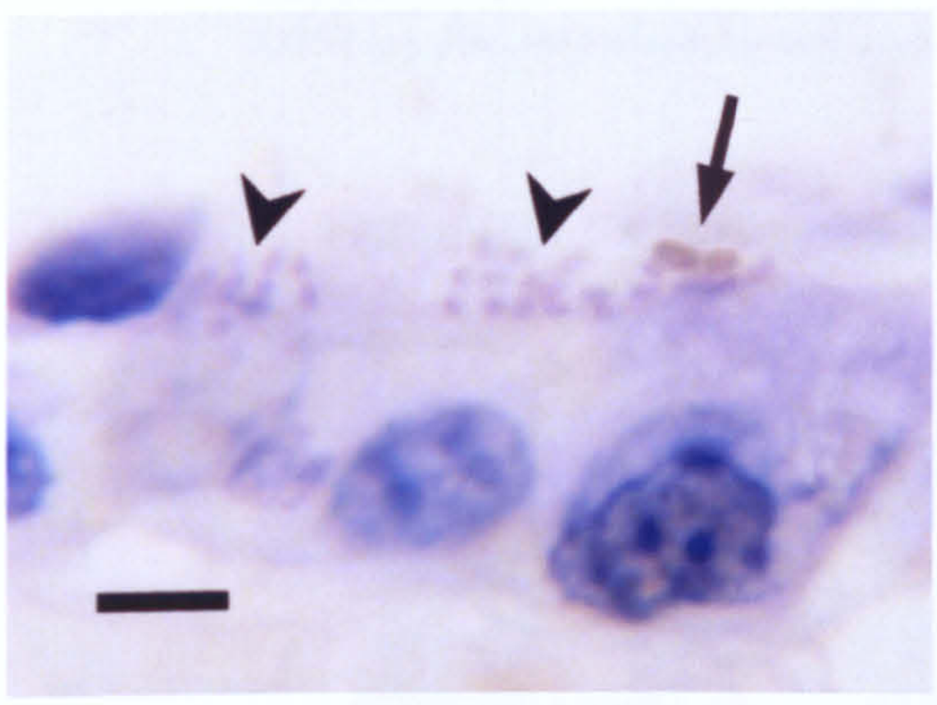
Lamb 29, caecum. Closely-adherent immuno-labelled organisms (arrow) are on the luminal surface of three adjacent enterocytes. O157 ipx, bar = 10 μm.

Figure 6-j: Attaching-effacing lesion with adjacent O157 bacteria



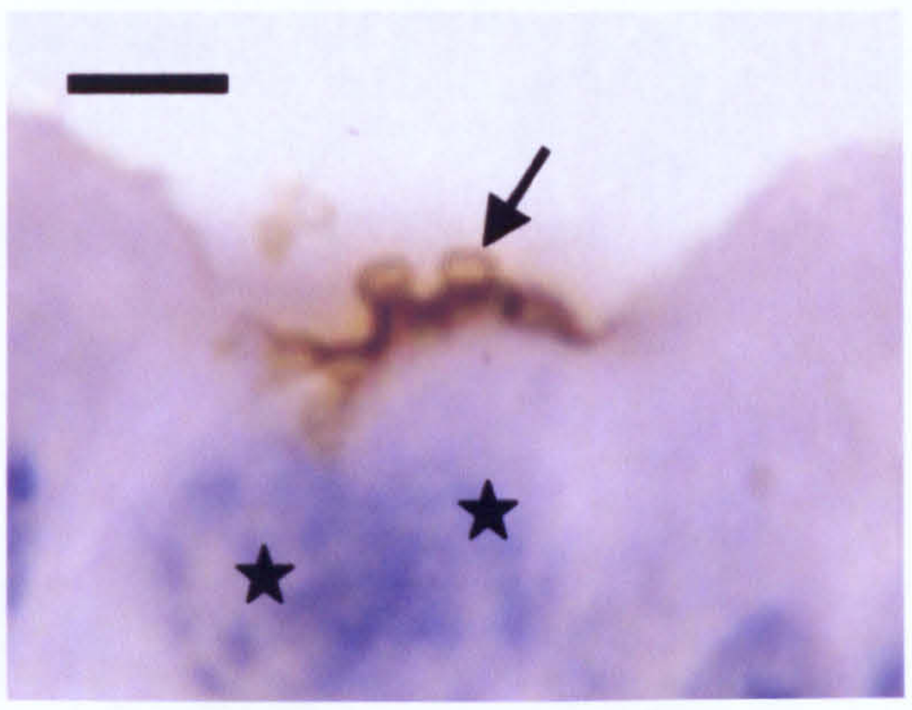
Lamb 29, caecum. A small cluster of immuno-labelled organisms (arrow) is adherent to the mucosa, adjacent to an unlabelled AE lesion (asterisk). O157 ipx, bar = 10 μm.

Figure 6-k: Possible mixed bacteria in attaching-effacing lesion



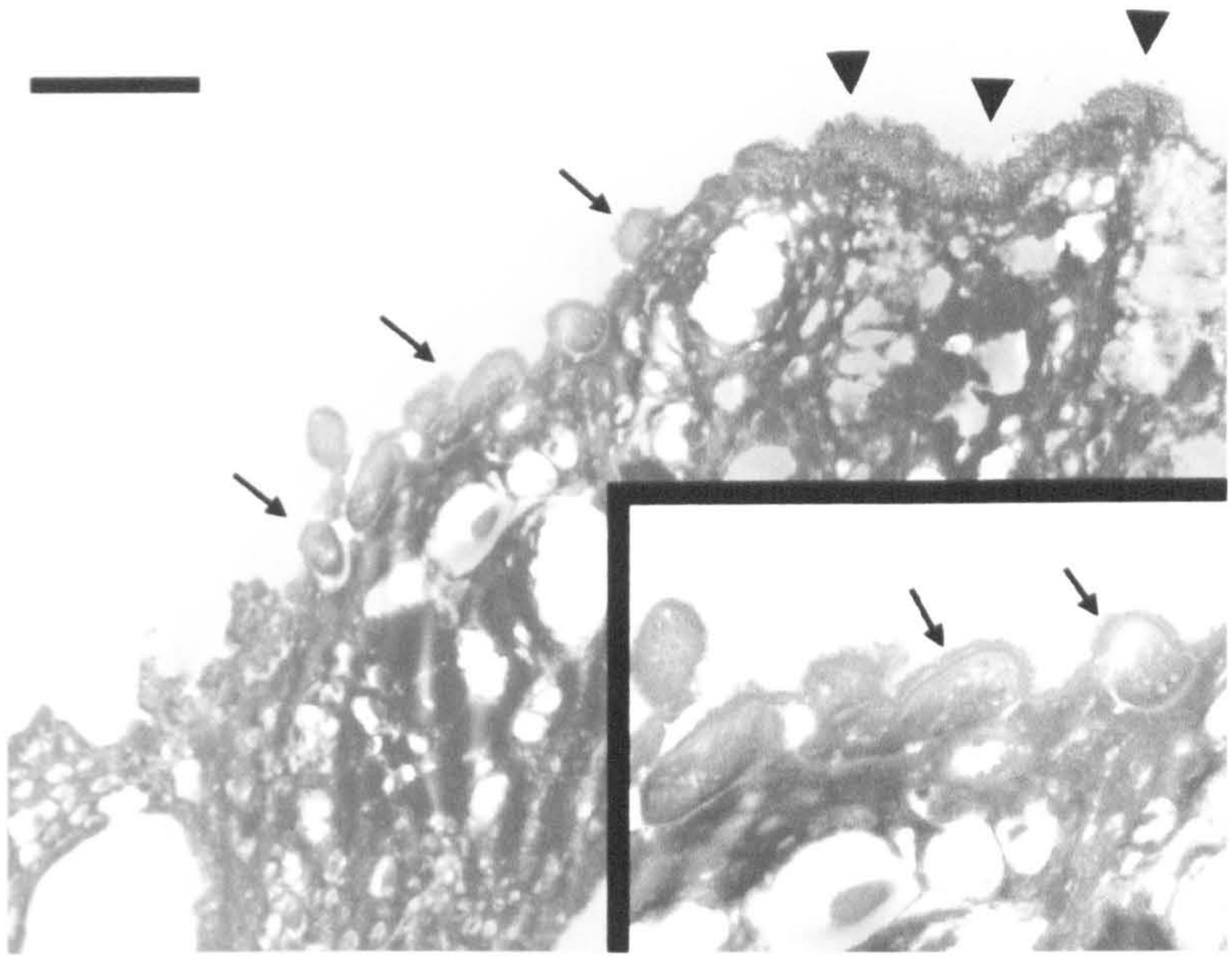
Lamb 29, rectum. Immunolabelled organisms (arrow) are present associated with non-labelled organisms (arrowheads) in an AE-type lesion. O157 ipx, bar = 5 μm.

Figure 6-l: *E. coli* O26 attaching-effacing lesion



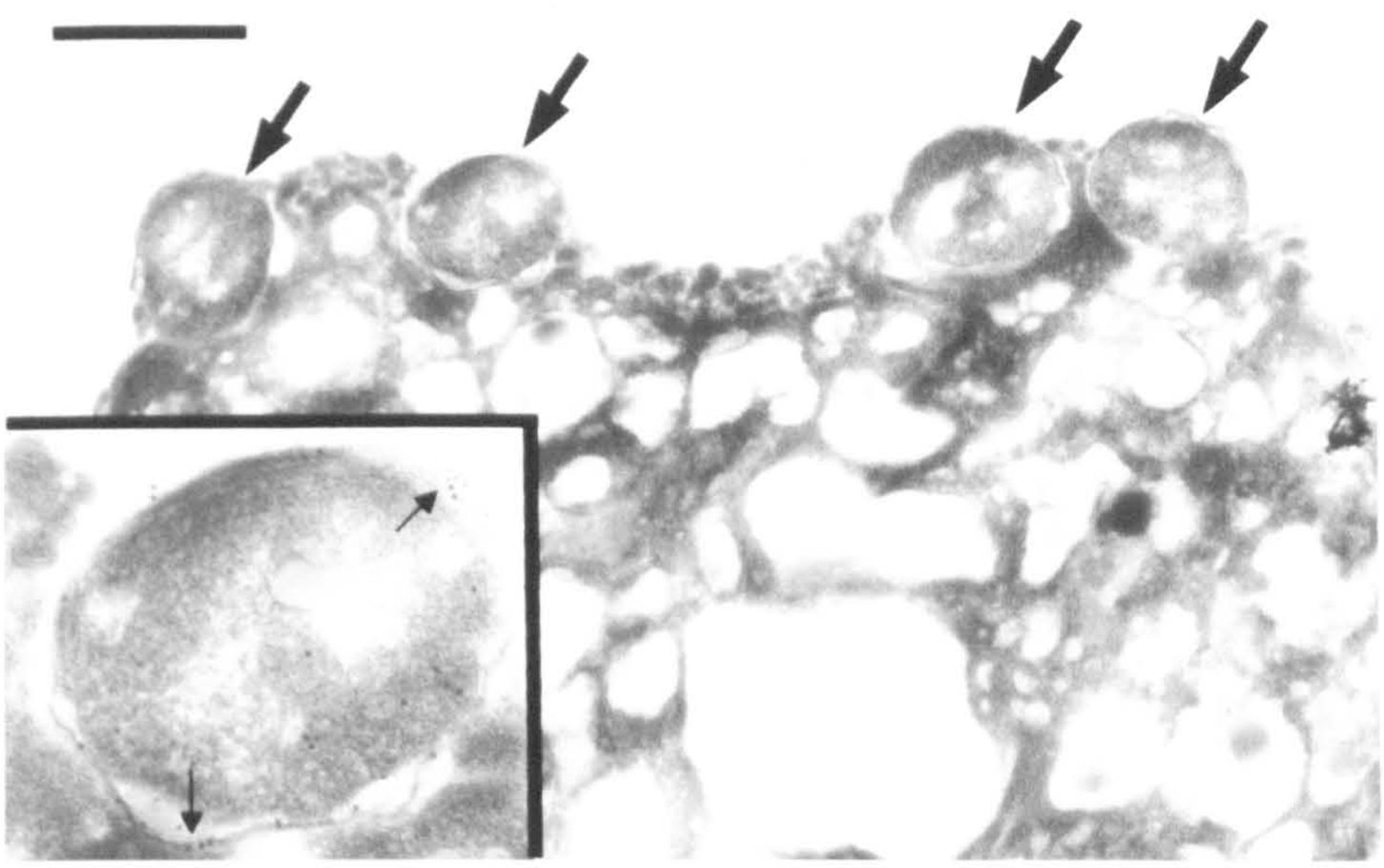
Lamb 29, caecum. A mass of immunolabelled organisms (arrow) is closely adherent to the luminal surface of two adjacent enterocytes (asterisks). O26 ipx, bar = 4 μm.

Figure 6-m: Ultrastructure of *E. coli* O157 attaching-effacing lesion



Lamb 29, caecum, 'popped-off' lesion previously shown in Figure 6-i. Adherent bacteria have formed AE lesions (arrows). Microvilli (arrowheads) have been effaced in the area of the lesion. Inset enlargement: Adherent bacteria have an irregular coating of presumed diaminobenzidine (DAB) reaction product.
O157 ipx plus lead citrate/uranyl acetate, bar = 3 μm.

Figure 6-n: Immunogold-labelled attaching-effacing lesion



Lamb 29, caecum. Four bacteria (arrows) have formed AE lesions. Inset enlargement: At higher magnification, immunogold labelling of the bacterial cell wall is evident (examples arrowed). O157 immunogold, bar = 750 nm.

Chapter 7 – GENERAL DISCUSSION

E. coli persistence

'Persistence' of *E. coli* in the intestines of mammals is not well defined. Some workers define 'majority' versus 'minority' strains, whilst others prefer to define 'resident' versus 'non-resident' or 'transient' strains (Hartl and Dykhuizen 1984; Hinton 1985). Work in humans and dogs suggests that 'resident' strains persist for several weeks to years in any individual whilst 'transient' strains disappear after days to weeks (Hartl and Dykhuizen 1984; Hinton 1985), so there appears potentially to be an overlap between the persistence categories. Bacterial factors that may enable the establishment of a persistent, or resident, status include: possession of certain fimbrial adhesins (Tullus et al. 1992; Nowrouzian et al. 2001) and of K1 and K5 capsules (Nowrouzian et al. 2001), colicin production by the organism and a high rate of growth in intestinal mucus (Wadolkowski et al. 1988). There is also evidence from murine studies that, once established in the intestine, an *E. coli* strain may have an increased ability to persist in competition with strains introduced subsequently (Wadolkowski et al. 1988).

Epidemiological studies on the excretion of *E. coli* O157:H7 by cattle (Section 1.4.2.6) suggest that the organism is not commonly isolated from any individual on consecutive (usually monthly) sampling occasions. However, from a small proportion of individuals the organism is recovered repeatedly, for three to eight consecutive months (Besser et al. 1997; Heuvelink et al. 1998b; Shere et al. 1998; Conedera et al. 2001; Jonsson et al. 2001). Therefore, the evidence indicates that *E. coli* O157:H7 does not normally become a long-term 'resident' strain in the gastrointestinal tract of cattle, although persistence of the order of a few months is detectable in some individuals, and persistence amongst the herd is typical (Section 1.4.2.6). Experimental studies have shown that detectable excretion of *E. coli* O157:H7 by yearling and young adult cattle and by sheep typically persists for one to two months after oral inoculation (Cray and Moon 1995; Kudva et al. 1995; Cornick et al. 2000; Wray et al. 2000; Cornick et al. 2002). Therefore, it seems reasonable to discuss the persistence of *E. coli* O157:H7 in ruminants as a phenomenon of excretion for several days or weeks to occasionally several months in any one individual. It is also reasonable to suggest that, in this respect, *E. coli* O157:H7 may not differ greatly from many other 'transient' *E. coli* strains (Hancock et al. 1998a).

However, the long-term persistence of *E. coli* O157:H7 within groups of cattle and sheep is a well-established feature of the serotype, and it may be hypothesised that the LEE-mediated capacity of *E. coli* O157:H7 to adhere to epithelial cells has a role in this (Gyles 1998). If a LEE-mediated attachment, either classical intimate attachment and/or perhaps a

non-intimate EspA-associated adherence (Tatsuno et al. 2000), could be shown to occur in the context of the colonisation of the intestine by *E. coli* O157:H7, it would indicate that, in persistently-excreting groups of animals, strategies of control which target LEE mechanisms may have some measure of success.

Consequently, the present studies examined the *E. coli* O157:H7 persistence phenomenon at the level of the individual animal, focussing in particular on events at the mucosal surface. Attempts were made also to correlate the behaviour of *E. coli* O157:H7 *in vivo* with features of the same strains *in vitro*.

Critique of methods

Lambs were inoculated orally with a mixture of strains from human and bovine sources, or with a single strain, at doses of between 5 x 10^8 cfu/lamb (Experiment 6/2, mixed) and 4 x 10^9 cfu/lamb (Experiment 6/4, single strain). Experimental inoculation in this fashion differs substantially from the likely process of field exposure to the organism, but it is an established and practical method for short- and medium-term studies, and it permits the comparison between individuals of the results of ingestion of a defined quantity of viable bacteria. The value of examining both human- and animal-derived strains is emphasised by findings that when strains from bovine and human sources were examined in respect of LEE protein secretion (McNally et al. 2001) or octamer-based subtyping (Kim et al. 1999), consistent differences between bovine- and human-derived strains were evident.

The chronological sequence of oral inoculation experiments in the present studies was: six-month old lambs with a mixed inoculum (Experiment 6/1), six-week old lambs with a mixed inoculum (Experiment 6/2), neonatal lambs with a mixed inoculum (Chapter 5), six-week old lambs with a single strain Shiga toxin-producing inoculum (Experiment 6/3), six-week old lambs with a non Shiga toxin-producing inoculum (Experiment 6/4). This order reflected a desire to use neonatal animals as a model to establish principle only if an effect (in this case mucosal adherence) was not detected in the older age groups that are more relevant to persistence. In the light of emerging results the emphasis of investigations shifted from multiple- to single-strain inoculations. Following the observation in Experiment 6/2 that faeces culture was often positive for *E. coli* O157:H7 whilst intestinal samples taken within hours were negative, the tissue sampling protocol was extended to include the intrapelvic rectum in the neonatal lamb study and in experiments 6/3 and 6/4.

A further mode of *in vivo* investigation of interactions between *E. coli* O157:H7 and host using ligated intestinal loops was prompted by the desire to make comparisons between multiple strains within a single animal and by the possibilities of readily examining host and bacterial responses *in situ,* as discussed in Section 4.1.

The combination of bacteriological and pathological approaches aimed to permit both detailed scrutiny of the mucosa and sensitive detection of the distribution and persistence of *E. coli* O157:H7 in the host. When examining the tissues of animals that were excreting variable and often low concentrations of the organism, pathological techniques needed to be sensitive to a potentially low incidence of bacterium-mucosal interactions. Therefore, tissue sampling protocols were used that aimed to place the intestinal mucosa rapidly in fixative, and which largely succeeded in providing high quality histopathological sections. Relatively large numbers (for example 98 large intestine sections from four animals in Experiment 6/2) of H&E-stained intestine sections were then examined from each animal. Ipx was initially used as an ancillary technique to verify the identity of lesions detected by H&E-stained sections. The value of ipx staining as a complementary technique to H&E was emphasised following examination of the lesions in the intestinal loop experiments, where a superior sensitivity of ipx over H&E was evident (Section 4.4), and following experiments 6/3 and 6/4, where non-O157 AE lesions frequently were present.

Where adherence of bacteria to the mucosa was seen, elucidation of the mechanisms of attachment required ultrastructural analysis by TEM. Conventional approaches to EM specimen preparation working from wet fixed tissue would have been insufficiently sensitive to obtain lesions in section, given the rarity and extremely small size of the lesions that eventually were detected. Therefore, LM-targeted recovery of the lesions from the associated wax block and, where this failed, from the histological slide, was employed as described in Section 2.7.4.2. The polysaccharide O157 antigen proved to be sufficiently robust to allow immunogold labelling of specimens prepared in this fashion.

In summary, the methods employed proved appropriate but areas for improvement are evident. In particular, the routine use of relatively time-consuming IMS could be replaced by a more direct TVC approach in most samples as the data in Figure 6-d shows, with IMS of pre-enrichment broths only employed if the TVC proved negative. Furthermore, if antibiotic resistance marked strains are being recovered, enrichment in broth containing the relevant antibiotic has proved even more sensitive than IMS (Besser et al. 2001), whilst being less labour-intensive. The apparent advantages in sensitivity and ease of examination of ipx- over H&E-stained slides indicate that, if facilities exist for the economic preparation of many ipx-stained sections, this technique should be preferred as the primary stain for screening intestinal tissues for *E. coli* O157:H7.

Comparison of findings from differing experimental approaches

Experiment 6/2 (*in vivo* oral inoculation) demonstrated a dichotomy in the persistence phenotype of the inoculated strains, showing that one (Strain 140065 nal^r) was clearly more persistent than the other three. The persistence of this strain was confirmed in a single-

inoculum experiment (6/3). This finding suggests that a view of *E. coli* O157:H7 strains as 'transient' (Hancock et al. 1998a) may be over-simplistic, as there appears to be substantial bacterially-determined variation in the level and length of excretion of *E. coli* O157:H7 by ruminants. Factors that logically may influence persistence include adhesins, including complex adhesion mechanisms such as the AE phenotype, and resistance to stresses of the gastrointestinal environment including acid pH and VFA.

The phenotype of *E. coli* O157:H7 strains *in vitro* and *in vivo* in ligated colon loops indicates that an AE capability cannot be the sole determinant of the persistence observed in the weaned lamb studies. Strains 218, 218 rif^r, 140065, 140065 nal^r, EC157, EC157 str^r and NCTC 12900 all readily formed AE lesions *in vitro*, both on HEp-2 and bovine intestinal cells (sections 3.5.2.1C and 3.5.2.2C). When strains 218 rif^r, 140065nal^r, EC157str^r and NCTC 12900 were tested *in vivo*, two of these (218 rif^r and EC157 str^r) were not persistently excreted. (Strain NCTC 12900 has been shown by other workers (La Ragione, R.L. personal communication) to be persistently excreted by weaned sheep.) Similarly, it was not possible to distinguish persistent from non-persistent *E. coli* O157:H7 strains on the basis of their ability, or that of their parent strains, to form AE lesions in ligated ovine colon loops (Table 4-B). Whilst a simple relationship between AE lesion-forming ability and persistence is not evident, it is certainly possible that an AE capability is important for the establishment of persistence.

The density of *E. coli* O157:H7 organisms in the samples from lambs where O157-associated AE lesions were found (neonatal lamb study and Experiment 6/4) is consistent with the suggestion by Dean-Nystrom et al. (1999) that a minimum density of approximately 10^6 cfu/g of *E. coli* O157:H7 is required for histopathological detection of lesions. Whether this apparent lower limit is an effect of the sensitivity of histopathological examination or because the formation of lesions is a density-dependent phenomenon, perhaps mediated by quorum sensing (Sperandio et al. 2001), is unclear.

Other *in vitro* phenotypic assessments, comparing all orally inoculated strains plus parent strains, yielded results that were not predictive of a strain's persistence behaviour in the orally inoculated weaned lambs. In secreted protein and intimin expression assays (Figure 3-c), in three-hour quantitative adhesion assays on HEp-2 (Figure 3-f) and bovine intestinal (Figure 3-j) monolayers, and in VFA tolerance assays (figures 3-l, 3-m, and 3-n) the persistent strain 140065 nal^r showed no notable phenotype in comparison with the other strains tested.

Whilst these findings do not help to predict a strain's persistence behaviour, they do not negate the possibility that intestinal adhesion or VFA tolerance are necessary, but not sufficient, for persistence. The findings of Cornick et al. (2002) do indeed suggest that intimin-mediated adhesion is significant in the persistence of *E. coli* O157:H7 in sheep and

cattle. Furthermore, phenotypes vary with environmental conditions leading to, for example, an increased adhesion of *E. coli* O157:H7 to HEp-2 cells when the bacteria are grown anaerobically (James and Keevil 1999). Under such conditions a phenotypic distinction between persistent and non-persistent strains might emerge. Another example of possible complex effects concerns bacterial resistance to acidic conditions. The resistance to inorganic acid at low pH of each of the parent strains of the mixed inoculum components (Table 3-K) appeared to have no discernible effect upon the level of each antibiotic resistance marked strain's initial excretion (Figure 6-c). Whilst this might suggest that the inorganic acid resistance phenotype of *E. coli* O157:H7 strains do not greatly affect the colonisation of the intestinal tract, there may be a different effect in the field, when oral intake of the organism is likely to be intermittent and at a lower level than in the present studies.

An *E. coli* O26:H11 strain (EC1537), which was associated with naturally-occurring AE lesions and enteritis in weaned cattle, was compared with *E. coli* O157:H7 strains *in vitro* and in ligated ovine colon loops. The O26 strain did not show more marked AE lesion formation on bovine intestinal cells (Figure 3-k) or on the intact ovine colonic mucosa (Table 4-B) than *E. coli* O157:H7. The pattern of lesion formation in the ligated colon loops differed between O157 and O26 strains, with evidence that *E. coli* O26:H11 formed lesions more readily without induction in DMEM than did most *E. coli* O157:H7 strains. However, when all the cell monolayer and colon loops data are considered together, it is difficult to conclude that, on bovine and ovine enterocytes, the 'non-pathogenic' *E. coli* O157:H7 formed AE lesions any less readily than did the 'pathogenic' *E. coli* O26:H11. This is subject to the caveat that the true pathogenic status of the *E. coli* O26:H11 EC1537 strain in cattle and sheep is unknown in the absence of oral challenge data, but it does suggest that the degree of AE adhesion to the intestinal mucosa in naturally-exposed animals may be substantially governed by factors that are not evident *in vitro* or in intestinal loop assays of AE capability. Similarly, in ligated colon loops of neonatal calves (Sandhu and Gyles 2002), *E. coli* O157:H7 also did not appear to adhere or form AE lesions any less readily than did any of three presumptive bovine-pathogenic Shiga toxin-producing AEEC (O5:H-, O26:H11, O111:H-) that had been isolated from diseased animals. In the present ligated loop study, meaningful statistical comparisons between the incidence of AE lesions formed by different strains were precluded by the scarcity of AE lesions, the tendency for clustering of lesions, the inherent variability in the length of epithelium present per section at the microscopic level and the possible variation in susceptibility to lesion formation within the spiral colon.

Examination of *E. coli* O157:H7 strains on cell monolayers and in ligated colon loops provided no evidence that Shiga toxin production altered a strain's propensity to form AE lesions, which is in agreement with findings from a ligated intestinal loop study in calves

(Sandhu and Gyles 2002). The data from the oral inoculation experiments is less clear, as the STEC-inoculated lambs of experiments 6/2 and 6/3, which did not have detectable O157-associated lesions, were tissue-sampled later post-inoculation than was the non-STEC-inoculated lamb in Experiment 6/4 which did have demonstrable lesions. One report (Dean-Nystrom et al. 1999) suggests there may be a positive effect of Shiga toxin on the density of *E. coli* O157:H7 achieved in the large intestine of orally inoculated weaned calves in the short term. Shiga toxin production might have a role in persistence, possibly by modulating local host immune responses. Shiga toxin has structural similarities with cholera toxin and ETEC labile enterotoxin (O'Brien and Holmes 1987). However, whereas cholera and labile toxins are potent adjuvants of mucosal immunity (Simmons et al. 2001), assays using Stx1 *in vitro* on human (Cohen et al. 1990) and bovine (Menge et al. 1999) B-lymphocytes, and *in vivo* in pigs (Christopher-Hennings et al. 1993), show selective immunosuppressive effects. Therefore, a role for Shiga toxin in suppressing a mucosal immune response to colonising *E. coli* O157:H7 is plausible.

The present oral inoculation studies demonstrate that the spiral part of the ovine ascending colon appears to be relatively resistant to the formation of AE lesions by *E. coli* O157:H7 and other AE organisms which form lesions spontaneously in sheep. Despite numerous AE lesions, formed by diverse AE organisms, having been detected in the large intestine (Chapter 5 and experiments 6/3 and 6/4) no such lesions were seen in any of the spiral colon tissues. However, the spiral colon is potentially susceptible to AE lesion formation, as AE lesions were observed in this location when, for technical reasons, it was used for ligated loop studies. Possibilities for mechanisms in the spiral colon that underlie this distribution include a relative lack of a mucosal surface receptor, a quantity or quality of mucus which prevents bacterial contact with enterocytes, and a luminal environment lacking appropriate signals for the up-regulation of the AE lesion-forming apparatus.

In the present studies, two ipx-stained AE-type lesions containing a mixture of immunolabelled and non-immunolabelled organisms were seen, one in a ligated loop (Figure 4-e) and one in an orally inoculated animal (Figure 6-k). It is not possible to say with confidence whether this uncommon feature genuinely illustrates 'mixed' AE lesions formed by both inoculated and endogenous AE bacteria or whether it is a product of incomplete labelling of O26 and O157 bacteria by the ipx technique. It is at least plausible that either or both illustrated lesions are mixed, as the ipx technique proved to be robust in labelling all organisms in all other positive lesions. However, an attempt to examine the lesion in Figure 6-k by TEM using the 'pop-off' technique was unsuccessful, and the issue remains unresolved.

E. coli O157:H7 persistence in sheep

In the present studies, the use of a mixed oral inoculum permitted the separation of four *E. coli* O157:H7 strains into one that was 'persistent' and three that were 'non-persistent' in weaned lambs. Under the conditions used, non-persistent strains were not excreted beyond eight days p.i. by any of the four weaned animals inoculated with them in Experiment 6/2, whilst the persistent strain was detected continuously in the faeces of four out of five weaned animals euthanased at between 17 and 28 days p.i., in experiments 6/2 and 6/3. The duration of excretion of the 'persistent' strain is in line with reports of the excretion of *E. coli* O157:H7 by sheep (Section 1.4.2.7), typically of one to two months. Therefore, it may be hypothesised that there are factors in *E. coli* O157:H7 that promote its persistence in the alimentary tract of sheep for periods of several weeks, and that these factors are subject to variations, possibly including selection for antibiotic resistance in the present studies, which alter a strain's persistence phenotype. Whether the persistence of *E. coli* O157:H7, and its range of variation, differs in degree from that of so-called 'transient' intestinal *E. coli* in sheep is unknown. One possible mechanism contributing to persistence is adherence to the intestinal mucosa. The intestinal loop (Chapter 4) and oral inoculation (chapters 5 and 6) studies demonstrate for the first time that *E. coli* O157:H7 does have a capacity to adhere to the ovine large intestinal mucosa, across an age range from neonates to six months old. There was no good evidence from the present studies that the organism adhered to the mucosa by any method other than AE lesion formation, although in ipx-stained sections of *E. coli* O157:H7-inoculated colon loops (Section 4.3, Lambs 3 and 4) there were scattered, solitary O157-bearing bacteria that were associated with the intestinal mucosa by an undetermined mechanism. These may however have been in the early stages of AE lesion formation. Where sections from orally inoculated lambs containing loosely-adherent bacteria and debris were examined by ipx (tables 5-C and 6-A to 6-C), there was no evidence of adherent O157-bearing bacteria.

The findings of the present studies suggest that there are mechanisms of colonisation possessed by some *E. coli* O157:H7 strains that can promote a state of intestinal persistence, in comparison with some other strains. The evidence also points to an AE capacity of *E. coli* O157:H7 in orally exposed weaned lambs. There is however no good evidence in the present data for a causal link between AE lesion formation and persistence, as lesions have not been detected in the context of persistent (i.e. for the present studies, greater than two week's) excretion, and there appears to be no simple relationship between a strain's tendency to form AE lesions, *in vitro* and in ligated spiral colon, and its persistence. Conversely, evidence from oral inoculation studies does suggest that the LEE, and therefore possibly AE adherence, affects the colonisation of the intestine by *E. coli* O157:H7. Dean-Nystrom et al. (1999) reported that nine weaned calves inoculated with a wild-type *E. coli* O157:H7 strain

had a greater density of the organism in the large intestine than did four calves inoculated with an isogenic intimin-deficient mutant. Cornick et al. (2002) showed that an intimin-deficient mutant appeared to be a poorer coloniser of sheep and cattle in respect of average duration of excretion. A finding by Magnuson et al. (2000), that crypt enterocyte proliferation indices in the large intestine of orally inoculated heifers were significantly negatively correlated with the duration of *E. coli* O157:H7 excretion, also suggests that the persistence of the organism may depend to some extent on interactions with the intestinal mucosa. A promising recent finding is that persistent *E. coli* O157:H7 appear to attach preferentially to the follicular epithelium at the recto-anal junction of calves (Naylor et al. 2003) by an as-yet undefined mechanism, which could be LEE-associated.

There is a further, unexpected, finding of the present studies which appears to be hitherto unreported and which demonstrates that when commensal AE organisms colonise the ovine intestinal tract, AE lesion formation does occur, at least in some cases. In four of 30 experimental lambs (13 %), AE lesions were seen in the large intestine of clinically normal animals, representing colonisation of the mucosa by *E. coli* O115 (Experiment 6/3), and by *E. coli* O26 and/or at least one other AE organism (Experiment 6/4). The affected animals were obtained from two flocks, separate in terms of geography and management. It might therefore be supposed that asymptomatic colonisation of the ovine mucosa by AE organisms is a relatively common phenomenon which has previously escaped recognition because extensive histopathological examination of adequately preserved intestinal mucosa from clinically normal sheep is not commonly performed. It might also be hypothesised that the demonstrable formation of many small AE lesions by these bacteria, which are apparently commensal in healthy weaned lambs, assists in their colonisation of the intestine, which is the same mechanism as has been hypothesised for *E. coli* O157:H7 (Gyles 1998). In experiments 6/3 and 6/4, sufficient numbers of the presumptive adherent strains were present in faeces to provide a detectable number of spontaneous nalidixic acid resistant mutants (Experiment 6/3) and to yield uncountable blue and colourless colonies on agar plates (Experiment 6/4). This strongly suggests that these lesion-forming commensals are excreted in substantial numbers by colonised lambs. It is of further interest that one of these spontaneous lesion-forming AE organisms appears to be an STEC O26. Some Shiga toxin-producing members of this serogroup are important human EHEC and are believed to have a bovine reservoir (Bettelheim 2000). STEC O26:H11 are also recognised as bovine pathogens (Mainil et al. 1987; Wray et al. 1989; Iijima et al. 1990; Janke et al. 1990; Pearson et al. 1999; Gunning et al. 2001).

Therefore, the findings of the present studies are consistent with the hypothesis that the formation of intestinal AE lesions by *E. coli* O157:H7 does have a role in the persistent colonisation of sheep by this organism. The *in vitro*, intestinal loop and mixed oral

inoculation studies together indicate that an AE lesion-forming capability is not the sole determinant of persistence, which is consistent with another report (Cornick et al. 2002) where an intimin-deficient *E. coli* O157:H7 mutant did persist, albeit in only one of eight cattle, for 60 days p.i. It may be that AE adhesion to the intestine, so far only detected acutely in cattle (Dean-Nystrom et al. 1999) and sheep (present studies) is only of importance early on in the process of persistent colonisation.

Wadolkowski et al. (1988) reported that one *E. coli* strain (F-18col-) was at a relative disadvantage to another (F-18) in its ability to colonise streptomycin-treated mice. However, F-18col- proved able to prevent colonisation by F-18 when the former was first permitted to colonise mice alone. This suggests that initial 'success' in intestinal colonisation, by whatever means, confers a competitive advantage over at least some other members of the intestinal flora, which supports the hypothesis that an initial (but possibly transient) event, such as the formation of AE lesions, may be important in the establishment of *E. coli* O157:H7 persistence. The dose of *E. coli* O157:H7 given orally to cattle and sheep appears to affect the duration of excretion in many cases, with doses of 10^{10} cfu being associated with the reliable induction of persistent excretion but lower doses, of 10^4 to 10^7 cfu, having a more variable outcome (Cray and Moon 1995; Kudva et al. 1995; Cornick et al. 2000). Again, therefore, an initial state (high density of *E. coli* O157:H7 in the intestine) appears to affect persistence. An initial high density of *E. coli* O157:H7 in the intestine of cattle is associated with a functional LEE (Dean-Nystrom et al. 1999; Cornick et al. 2002). The evidence on the role of the initial density of organisms is complicated by the finding that some *E. coli* O157:H7 strains will establish persistence in calves, and transmit to others, following low inoculum doses, of the order of 200-300 cfu per animal (Besser et al. 2001). These strains appear to colonise gradually, with low levels of the organism appearing in the faeces initially. A high luminal density may be helpful for successful colonisation but it does not appear to be an absolute prerequisite.

It is possible that the LEE functions at both the group and the individual level to promote persistence. A short-term effect of the LEE in increasing the number of organisms excreted following ingestion may increase the level of environmental exposure to the organism experienced by members of the herd or flock. This may in turn promote persistence within a group.

The lack of AE lesions demonstrated to date in the large intestine of persistently excreting ruminants may be explained by the recent report that *E. coli* O157:H7 appear to adhere preferentially to the recto-anal junction of cattle and are transferred to the faeces as they are passed (Naylor et al. 2003). It is postulated in that report that recto-anal colonisation may be responsible for the few animals which excrete *E. coli* O157:H7 persistently at high levels, and who may be significant in the maintenance of the organism in any group of animals. In

the present studies, only one animal (Lamb 26, Figure 6-d) appeared to fit this 'super-shedder' category and when its intestinal tract was examined, colonisation throughout the large intestine was found, i.e. the data supports a role for extensive rather than focal large intestinal colonisation in this animal. When it is appreciated that a 2 cm x 4 μm histopathological section represents approximately 0.0007 % (0.008 cm^2 / 1201 cm^2, Appendix 5) of the epithelial surface area of the ovine large intestine, it is certainly possible that sparse *E. coli* O157:H7 AE lesions will be missed, even with extensive tissue sampling and the examination of multiple sections.

There is much variation, in the present studies and in other reports (Kudva et al. 1995; Cornick et al. 2000; Magnuson et al. 2000; Grauke et al. 2002; Naylor et al. 2003), in the alimentary tract location and in the observed level and duration of excretion of *E. coli* O157:H7 in orally inoculated ruminants. The possible host factors of diet and intestinal epithelial turnover have been considered elsewhere (Kudva et al. 1995; Kudva et al. 1997b; Cray et al. 1998; Harmon et al. 1999; Tkalcic et al. 2000; Grauke et al. 2002), but the present findings have raised another possible influence, that of pre-existing AE lesions. Thirteen percent of animals in the present studies had detectable non-O157 AE lesions in the large intestine, and in another study 25% of ovine faecal *E. coli* isolates encoded *eaeA* (Orden et al. 2000). These findings suggest that AE lesions may be widespread in asymptomatic animals and that the opportunities for interactions between *E. coli* O157:H7 and other AE bacteria in sheep are likely to be plentiful. Furthermore, the observation of an otherwise comparatively rare O157 lesion in close proximity to a non-O157 AE lesion (Figure 6-j) and a possibly co-incident O157 plus non-O157 lesion (Figure 6-k) prompts speculation that the attachment of *E. coli* O157:H7 to the large intestinal mucosa may be facilitated by pre-existing AE lesions. Conversely, it might be hypothesised that pre-existing AE lesions might have a negative influence on the ability of *E. coli* O157:H7 to attach to the mucosa, perhaps by stimulating cross-reactive local immunity to LEE components, or by increasing enterocyte turnover.

Issues of immunological priming and responses were not addressed in the present studies, except for the precautionary screening of experimental animals for excretion of *E. coli* O157. An attempt to characterise an anti-O157 humoral response of cattle colonised by *E. coli* O157:H7 did not produce clear results (Wray et al. 2000), and detailed studies of systemic and local immune responses to persistent colonisation of animals by *E. coli* O157:H7 are lacking. Where sheep have repeatedly been inoculated with *E. coli* O157:H7, there is no evidence that recent prior exposure affects the excretion of the organism (Kudva et al. 1997b).

The present studies have indicated that, like cattle, persistence of *E. coli* O157:H7 excretion may be observed for periods of several weeks. Apparent differences between cattle

and sheep have also been seen. The extent of AE lesion formation in CF neonatal lambs was much less than in similarly-inoculated CF calves, as discussed in Section 5.4. However, the differences between age at inoculation and between the strains used limits useful comparison between these two studies. The absence of detectable *E. coli* O157:H7 in the rumens of persistently-excreting lambs in experiments 6/2 and 6/3 contrasts with the frequent detection of the organism in the rumens of persistently-excreting cattle of a similar age (Cray and Moon 1995; Brown et al. 1997; Harmon et al. 1999; Tkalcic et al. 2000). Such a difference may reflect differences in the ovine versus the bovine rumen environment, but it might also be associated with behavioural differences whereby calves may continually re-ingest higher numbers of *E. coli* O157:H7 from the experimental environment than do weaned lambs.

Future directions

Cell monolayer and intestinal loop studies, using simultaneous and sequential dual-inoculation of AEEC would be appropriate initial approaches to questions, raised in the present discussion, regarding the co-localisation of AEEC, including *E. coli* O157:H7, in lesions. In view of the distribution of AE lesions observed in the present lambs, it would be interesting, if technically demanding, to perform comparative studies in ligated segments of caecum and proximal and terminal colon, in addition to spiral colon, to see if a regional tropism for AE lesions could be demonstrated for a range of AEEC. Furthermore, the apparent bacterial concentration-dependence of detectable AE lesions could be addressed by the inoculation of a range of bacterial doses into ligated loops followed by assessment of the luminal density achieved by quantitative culture of the luminal contents and mucosa when the loops are harvested. Inclusion of the *E. coli* O26:H11 EC1537 strain in further intestinal loop experiments may clarify or negate the apparent differences between it and *E. coli* O157:H7 in terms of its propensity to form AE lesions under certain conditions.

Having identified diversity amongst the ovine persistence phenotype of *E. coli* O157:H7 strains, further studies using mutants of persistent strains that are deficient in LEE-mediated adherence could be used to clarify further the role of the LEE in the level and duration of excretion. The contribution of each of the two established LEE-mediated adhesins (intimin and EspA) to persistence could be examined by the use of appropriate mutants. To this end, a study using an *eaeA* mutant in Strain NCTC12900 has been performed at VLA Weybridge using the orally inoculated weaned lamb model (unpublished data) which demonstrates a statistically significant reduction in the level of excretion of the mutant in comparison with the wild-type. Dual-inoculation of animals with wild-type and mutant strains helps to control for inter-animal variation and reduces the number of animals required for statistical significance. However, such an approach also includes competition between inoculated strains as well as colonisation *per se*. Comparison should therefore also be made with

animals inoculated with a single strain. Studies using persistently excreting sheep should also seek evidence for preferential colonisation of the recto-anal area, as has been demonstrated in cattle. It is imperative that marked strains be assessed as fully as possible for AE capability *in vitro*, in comparison with parent strains. The use of non-toxigenic strains potentially permits more extensive investigations than do STEC, as the burden of microbiological containment with such strains is less severe than for STEC O157:H7.

Conclusions

The present studies have attempted to advance the understanding of *E. coli* O157:H7 persistence in sheep across a broad front, investigating a number of strains, comparing them with other reference AEEC, and evaluating several pathological techniques for the *in vivo* study of bacteria-host interactions. It can be concluded that the persistence phenotype of *E. coli* O157:H7 strains in sheep does vary and that *E. coli* O157:H7 can form AE lesions in the large intestine of weaned lambs. However, the role of such lesions, and of other AE organisms, in persistence remains undefined. It appears that colonisation, perhaps by AE lesion formation, of the recto-anal area may be a significant mechanism of persistence in cattle (Naylor et al. 2003). Evidence from Naylor et al. (2003) and from the present studies shows that more extensive colonisation of the large intestine by *E. coli* O157:H7 occurs in some calves and lambs, including in some high-level excreters. Extensive AE lesion formation in the large intestine, which has been shown in the present studies to occur for other commensal AE organisms, is one possible mechanism for this. Therefore, evidence from the present studies and other reports suggests that intervention in LEE-mediated adherence may reduce the persistent excretion of AEEC, including serotype O157:H7, by individual sheep and within flocks.

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Appendices

Appendix 1 – Alphabetical list of standard laboratory solutions and reagents

AEC chromogenic substrate

3-amino-9-ethylcarbazole (Sigma)	20 mg tablet
Dimethylsulphoxide (DMSO, Sigma)	12 ml
When dissolved, added to:	
Sodium acetate buffer (pH 5.4)	100 ml
Hydrogen peroxide	40 µl, added immediately before use

Agarose gel (0.8 %)

Agarose powder (Promega) 8 g
1x TAE buffer to 1000 ml
The powder was dissolved by heating the mixture in a loosely-capped glass bottle (Duran) within a pressure cooker at 121 °C for 15 min. The gel mixture was stored at 60°C until cast. Gels were cast in Perspex trays using appropriately-sized combs (BioRad) to form wells. When set, gels were immersed in 1x TAE buffer in a DNA Sub-Cell electrophoresis tank (BioRad).

CAPS buffer 10x concentrate

3-(cyclohexylamino)-1-propanesulphonic acid (Sigma) 22.13 g
Sterile distilled water to approx. 900 ml
pH adjusted to 11.0 with concentrated aqueous sodium hydroxide
Sterile distilled water to 1000 ml

The working solution of CAPS buffer was made with one part 10x CAPS buffer, one part methanol and eight parts deionised water.

Cell culture medium - complete

Foetal calf serum (Gibco)	10 %
L-glutamine 200mM (Sigma)	1 %
Non-essential amino acids, 100x (Sigma)	1 %
Gentamicin (Sigma)	0.5 %
Eagle's minimum essential medium (Sigma) to	100 %

Cell culture medium - incomplete

1 % L-glutamine (Sigma)	1 %
Non-essential amino acids (Sigma)	1 %
Mannose (Sigma)	1 %
Eagle's minimum essential medium (Sigma) to	100 %

Coomassie Brilliant Blue reagent

Brilliant Blue R (Sigma)	0.46 g
Methanol	160 ml
Glacial acetic acid	90 ml
Distilled water	200 ml

Coomassie destaining solution

Methanol	50 ml
Glacial acetic acid	100 ml
Distilled water	850 ml

Dorset's egg medium (prepared by VLA media section)

Contents of washed grade A hen eggs 750 ml
Physiological saline to 1000 ml
The mixture was homogenised, passed through a muslin filter, aliquoted in 3ml volumes into bijoux and placed on a gradient in an oven set at 75 °C for 2.5 hours. After cooling, bijoux were stored at 4 °C.

Luria-Bertani minus glucose (LB-G) broth (prepared by VLA media section)

Sodium chloride	5 g
Pancreatic digest of casein	10 g
Yeast extract	5 g

Sterile distilled water to 1000 ml
The mixture was adjusted to pH 7.5 with aqueous sodium hydroxide and sterilised by autoclaving for 15 min at 121°C (15 psi).

Neutral buffered formalin 10% (prepared at VLA and Langford by pathology technical staff)

Formalin (40% aqueous solution of formaldehyde)	100 ml
Sodium dihydrogen orthophosphate (monohydrate)	4 g
Disodium hydrogen orthophosphate (anhydrous)	6.5 g

Distilled water to 1000 ml

Nutrient agar (prepared by VLA media section)

Agar	15 g
Peptone	5 g
Sodium chloride	5 g
Yeast extract	2 g
Beef extract	1 g

Sterile distilled water to 1000 ml
The mixture was gently heated to boiling and sterilised by autoclaving for 15 min at 121°C (15 psi).

PCR loading buffer

Bromophenol blue (Sigma)	0.25 % w/v
Glycerol	30.0 % v/v
Sterile distilled water to100 %	

Phosphate-buffered saline (Prepared by VLA media section)

Solution A

Disodium hydrogen phosphate	5.93 g
Sodium chloride	4.25 g
Sterile distilled water to 1000 ml	

Solution B

Potassium dihydrogen orthophosphate	4.53 g
Sodium chloride	4.25 g
Sterile distilled water to 1000 ml	

Solution A was added to Solution B until a pH of 7.2 was achieved. The mixture was sterilised by autoclaving at 121 °C (15 psi) for 15 min, and stored at 4 °C.

Polyacrylamide separating gel (12 %)

30 % acrylamide solution (Protogel, National Diagnostics)	4.12 ml
4x separating gel buffer	2.5 ml
Sterile distilled water	3.25 ml
Tetramethyl ethylene diamine (Temed, Sigma)	10 µl
Ammonium persulphate 10 % aqueous	100 µl

Polyacrylamide stacking gel (5 %)

30 % acrylamide solution (Protogel, National Diagnostics)	1.3 ml
4x stacking gel buffer	1.9 ml
Sterile distilled water	4.2 ml
Tetramethyl ethylene diamine (Temed, Sigma)	7.5 µl
Ammonium persulphate 10 % aqueous	75 µl

SDS-PAGE 4x stacking gel buffer

Trizma base (Sigma)	60.5 g
Sterile distilled water to approx. 900 ml	
pH adjusted to 6.8 with concentrated hydrochloric acid	
Sterile distilled water to 1000 ml	

SDS-PAGE 4x Separating gel buffer

Trizma base (Sigma)	181.6 g
Sterile distilled water to approx. 900 ml	
pH adjusted to 8.8 with concentrated hydrochloric acid	
Sodium dodecyl sulphate (Sigma)	4 g
Sterile distilled water to 1000 ml	

SDS-PAGE 10x running buffer

Trizma base (Sigma)	30.3 g
Glycine	144.0 g
Sterile distilled water to c. 900ml, check pH, should be 8.3	
Sodium dodecyl sulphate (Sigma)	10.0 g
Sterile distilled water to 1000 ml	

SDS-PAGE loading buffers

i)

4x stacking gel buffer	2.5 ml
10 % SDS	4.0 ml
Glycerol	2.0 ml
Bromophenol blue	2.0 mg
Dithiothreitol	0.31 g
Sterile distilled water to 10 ml	

Stored at –20 °C for a maximum of six months.

ii)

Glycerol	50 % v/v
Bromophenol blue (Sigma)	0.02 % w/v
Sterile distilled water to 100 %	

2-mercaptoethanol added to sample/buffer mix at 5 % of final volume

Sheep's blood agar 5% (prepared by VLA media section)

Agar	15 g
Pancreatic digest of casein	15 g
Papaic digest of soybean meal	5 g
Sodium chloride	5 g
Sterile distilled water to 950 ml	

Mixed thoroughly and gently heated until boiling, then sterilised by autoclaving for 15 min at 15 psi (121 °C). The agar was cooled to 45-50 °C and then 50 ml of sterile sheep's blood was aseptically added, mixed in thoroughly and poured into sterile Petri dishes in 20 ml volumes and stored at 4 °C.

Sodium acetate buffer, pH 5.4

Sodium acetate	82.02 g
Sterile distilled water to 1000 ml	

TAE buffer 50x stock concentrate

Trizma base (Sigma)	242 g
Glacial acetic acid	57.1 ml
Disodium ethylene diamine tetraacetic acid, 0.5 M (pH 8.0, Sigma)	100 ml
Sterile distilled water to 1000 ml	

A working solution was prepared by diluting the stock 1 in 50 with distilled water.

Tris-buffered saline 10x stock concentrate

Trizma base (Sigma) 24.2 g
Sodium chloride 292.2 g
Sterile distilled water to approx. 900 ml
pH adjusted to 7.8 with concentrated hydrochloric acid
Sterile distilled water to 1000 ml
A working solution was prepared by diluting the stock 1 in 10 with distilled water.

Tris-EDTA buffer

Trizma base (Sigma) 1.21 g
Disodium ethylene diamine tetraacetic acid (Sigma) 0.37 g
Sterile distilled water to approx. 900 ml
pH adjusted to 8.0 with concentrated hydrochloric acid
Solution made up to 1000 ml with sterile distilled water

Appendix 2 – Alphabetical list of laboratory procedures and protocols

Electron microscopy heavy metal stains

Epoxy resin thin sections

Uranyl acetate (methanolic)	10 min
Lead citrate	5 min

Acrylic resin thin sections

Uranyl acetate (aqueous)	5 min
Lead citrate	5 min

All staining solutions were filtered through Millipore filters before use.
Filtered distilled water, or 50 % methanol then distilled water if using methanolic uranyl acetate, was used to wash between stains.
Care was taken not to breathe on the lead citrate whilst staining to avoid a precipitate of lead carbonate forming and contaminating the sections

Uranyl acetate:
Methanolic - saturated uranyl acetate in 50 % methanol.
Aqueous - saturated uranyl acetate in distilled water.

Reynold's lead citrate:
1.33 g lead nitrate.
1.76 g sodium citrate.
30 ml distilled water.
Shake for 1 min.
Allow to stand for 30 min shaking the solution occasionally.
Add 8 ml 1 M sodium hydroxide and mix.
Dilute to 50mls with distilled water.
Final pH should be pH 12.

Intimin and EspA antiserum production (performed by Dr A.L. Cookson et al.)

Purified *eae*A PCR products (962 base pair fragment at the 3' end of the gene, plus restriction sites added to primers) from *E. coli* O157:H7 strain A84 and pET21d were cut with *Bam*HI and *Hin*dIII and ligated overnight at 16 °C. *E. coli* DH5α (Life Technologies) were transformed with 2 µl of the ligation mix. White colonies were transferred from ampicillin / X-Gal transformation plates and cultured overnight in LB broth at 37 °C. Purified plasmids were digested with *Bam*HI and *Hin*dIII to ensure the correct insert size. The pET21d construct containing the 962 base-pair *eae*A inserts was used to electroporate *E. coli* BL21 (DE3) in order to express the recombinant intimin C-terminal 310 amino acids, now with a six-histidine tag derived from the expression vector.

The same procedure was used to generate a HIS-tagged recombinant EspA product comprising almost all of the EspA protein from *E. coli* O157:H7 strain EC157.

A 1ml aliquot of an overnight culture of the HIS-EaeA or HIS-EspA recombinant construct was inoculated into 200ml LB-broth containing ampicillin and incubated with

shaking at 37 °C for 4 hrs. Expression of the HIS-EaeA / HIS-EspA recombinant protein was induced by the addition of 1 mM IPTG and the culture was incubated for a further 2 to 3 hrs. Further recombinant protein purification, prior to injection into rabbits was performed using a nickel affinity column. Polyclonal antisera was prepared from New Zealand White rabbits by injecting equal amounts of recombinant protein preparation and Freund's complete adjuvant (100µl injected subcutaneously at 2 sites). Boosting with recombinant preparation antigen was performed at two-weekly intervals. Serum obtained was absorbed repeatedly with a plasmid-free BL21 (DE3) strain. Strains for absorption were grown on nutrient agar plates overnight at 37 °C (2 agar plates per ml of serum), harvested and incubated together with serum at 37 °C for 2 hrs with constant mixing. After each incubation the serum was centrifuged, the pellet discarded and another batch of BL21 (DE3) cells added. After each absorption the serum was titrated by ELISA using plasmid-free BL21 (DE3) and induced BL21 (DE3) containing the recombinant plasmid and the titre compared with that of non-absorbed serum.

Miles and Misra method for viable count determination

A 20 µl aliquot was removed from the bacterial suspension to be measured and was diluted tenfold by addition to 180 µl of PBS in a microtitre plate well. A serial tenfold dilution series was prepared in PBS on the microtitre plate. Depending upon the likely viable count, dilutions down to 10^4 to 10^6 were prepared. From each of the three or four most dilute suspensions, three 20 µl aliquots were plated onto labelled, partially-dried nutrient agar plates for overnight culture. After incubation, the least dilute suspension for which clear, separated colonies could be counted was used for colony counting, and an average number of colonies per 20 µl spot was determined from the three spots. The viable count of the original suspension was derived by multiplication using the dilution factor.

Stains: Giemsa for paraffin wax sections

i. Sections dewaxed in Histo-Clear (National Diagnostics) for 6 min minimum
ii. Hydrated through two baths of 100 % alcohol (Industrial Methylated Spirits) and one of 70 % alcohol, to tap water
iii. Stained in a coplin jar in a mixture of 1 ml of Giemsa stock* plus 45 ml distilled water in a water bath at 56 °C – 20 min to 1 hour (maximum)
iv. Rinsed in distilled water
v. Differentiated in 1/1,500 acetic acid for approx. 30 seconds total, rinsed in distilled water. (Controlled by viewing at intervals under a microscope. Sections should have an overall pink colour, with the nuclei blue and eosinophil granules red.)
vi. Rinsed in distilled water
vii. Blotted dry, rinsed briefly in alcohol, cleared in Histo-Clear and mounted under coverslips with DPX mountant (R. A. Lamb)

* Prepared as follows: Mix 7.36 g Giemsa powder in 500 ml glycerol which is heated to 50 °C in a water bath. Leave for 30 min at 50 °C with periodic mixing. Allow to cool and add 500 ml methanol. Mix and filter.

Stains: Gram-Twort

i. Dewaxed sections in Histo-Clear (National Diagnostics) for 6 min minimum
ii. Hydrated through two baths of 100 % alcohol (Industrial Methylated Spirits) and one of 70 % alcohol, to tap water
iii. Stained with Lillie's crystal violet – 1 min (filter stain onto slide)
iv. Washed briefly in tap water
v. Lugol's iodine – 1 min
vi. Drained the slide and flood with acetone to differentiate for 2-5 seconds
vii. Rinsed in distilled water
viii. Counterstained with neutral red/fast green – 5 min
ix. Washed in distilled water
x. Dehydrated, through 70 % and 2 x 100 % alcohol baths, to Histo-Clear. The alcohol step was prolonged until neutral red stopped coming out.
xi. Mounted under coverslips with DPX mountant (R. A. Lamb)

Lillie's crystal violet
Crystal violet – 2 g
Ammonium oxalate- 0.8 g
95 % alcohol – 20 ml
Distilled water – 80 ml
Dissolve the dye in the alcohol, add the rest, stir for 3 hours and filter.

Lugol's iodine
Iodine – 1 g. Dissolve it in a small quantity of the water then add the remainder.
Potassium iodide – 2 g
Distilled water – 100 ml

Neutral red/fast green solution
9 ml of 0.2 % neutral red in ethanol
1 ml of 0.2 % fast green in ethanol
30 ml of distilled water
Mix and use immediately

Stains: Haematoxylin and eosin

i. Sections dewaxed in Histo-Clear (National Diagnostics) for 6 min minimum
ii. Hydrated through two baths of 100 % alcohol (Industrial Methylated Spirits) and one of 70 % alcohol, to tap water
iii. Stained in Harris' haematoxylin for 10 min and wash in tap water
iv. Differentiated in acid alcohol (1 % HCl in 70 % industrial methylated spirits) for ten seconds and washed in tap water
v. Sections blue'd in Scott's tap water substitute for 45 seconds and washed in tap water
vi. Stained in eosin (1 % eosin Y {yellowish} in tap water) for 4 min and washed briefly in tap water
vii. Dehydrated, through 70 % and 2 x 100 % alcohol baths, to Histo-Clear
viii. Mounted under coverslips with DPX mountant (R. A. Lamb)

Stains: Immunogold

i. Grids placed in distilled water for 10 min
ii. Incubated in pH 7.4 TBS (Tris buffered saline) containing 5 % normal goat serum for 30 min
iii. Incubated in specific primary antibody (rabbit anti-O157, VLA) diluted 1 in 5 with pH 7.4 TBS, including 0.1 % bovine serum albumin, for 30 min. For negative controls, normal rabbit serum (Sigma) was used.
iv. Grids washed in two changes of pH 7.4 TBS for 5 min each, then two changes of pH 8.2 TBS for 5 min each
v. Incubated with secondary antibody (10 nm gold-conjugated goat anti-rabbit IgG, BBI) diluted 1 in 50 with pH 8.2 TBS, including 0.8 % bovine serum albumin, for 1.5 hours
vi. Grids washed in pH 8.2 TBS for 5 min x 2
vii. Grids post-fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer for 15 min
viii. Washed in two changes of distilled water for 5 min each
ix. Grids stained with uranyl acetate and lead citrate

Stains: Immunoperoxidase

i. Sections dewaxed by soaking in Histo-Clear (National Diagnostics) for at least 6 min, then rinsed in 100 % alcohol (Industrial Methylated Spirits)
ii. Endogenous peroxidase activity quenched by soaking in 0.5 % v/v hydrogen peroxide in methanol for 30 min at room temperature
iii. Sections washed and hydrated by soaking in PBS, pH 7.4, for 5 min x2
iv. Non-specific binding sites of bridge antibody blocked by applying normal goat serum (Sigma) diluted 1:5 in PBS and left in humid chamber for 30 min
v. Primary antibody applied to sections, appropriately diluted in PBS, and left in humid chamber at room temperature for 30 min
vi. Washed with PBS, 5 min soak x2
vii. Goat anti-rabbit secondary antibody (Sigma) diluted 1:50 in PBS applied and left in humid chamber at room temperature for 1 hour
viii. Washed with PBS x2
ix. Rabbit PAP complex (Dako) diluted 1:100 in PBS applied and left in humid chamber at room temperature for 30 min
x. Washed with PBS x3
xi. The chromogen diaminobenzidine (DAB, Sigma) 0.06 % w/v in PBS applied, with 0.03 % v/v hydrogen peroxide added at the last moment, for 1 min
xii. Colour development stopped with distilled water and sections counterstained in Mayer's haematoxylin for 30 seconds
xiii. The haematoxylin was blue'd in tap water for 5 min, sections were dehydrated through 70 % and 100 % alcohol, to Histo-Clear
xiv. Sections mounted under coverslips with DPX (R. A. Lamb)

Stains: Warthin-Starry

Using organo-silane (Sigma) treated slides. Glassware pre-washed with 10 % nitric acid followed by distilled water. Positive control section used. Plastic forceps used.

i. Sections dewaxed in Histo-Clear (National Diagnostics) for 6 min minimum
ii. Hydrated through two baths of 100 % alcohol (Industrial Methylated Spirits) and one of 70 % alcohol, to tap water
iii. Rinsed in pH 3.7 buffer
iv. All solutions prepared and heated to 56 °C in a waterbath
v. Slides placed in the 1 % silver nitrate solution at 56 °C for 1 hour
vi. 2.5 ml of the 2 % silver nitrate solution added to the gelatine solution
vii. The 1 % silver solution was tipped out leaving the slides in the coplin jar
viii. 3 ml of the hydroquinone added to the silver/gelatin mixture and poured into the coplin jar with the slides
ix. Slides treated in this solution until the sections were golden brown. This took 1-3 min.
x. Slides washed carefully in warm tap water
xi. Dehydrated, through 70 % and 2 x 100 % alcohol baths, to Histo-Clear
xii. Mounted under coverslips with DPX (R. A. Lamb)

pH 3.7 buffer

0.2 M Sodium acetate	1.5 ml
0.2 M Acetic acid	18.5 ml
Distilled water	480 ml

1 % Silver Nitrate in buffer

2 % Silver Nitrate in buffer

Gelatin Solution
3 g gelatine in 45 ml buffer

Hydroquinone
0.3 g hydroquinone (quinol) in 7 ml buffer

Tissue retrieval from wax blocks for electron microscopy

All steps to embedding were performed in glass vials on a rotating mixer.

i. The tissue was placed into a glass processing vial filled with Histo-Clear (National Diagnostics) and left for 24 hours.

ii. Tissue placed into 100 % ethanol for 2 changes of 1 hour each

iii. Tissue placed into 90 % ethanol for 2 changes of 30 min each

iv. Tissue placed into 0.1 M sodium cacodylate buffer for 2 changes of 30 min each

v. Post fixed in osmium tetroxide in 0.2 M sodium cacodylate buffer for 1 hour (Equal quantities of 2 % aqueous OsO4 and 0.4 M buffer mixed and used immediately)

vi. Rinsed in 0.2 M buffer for 2 changes of 5 min each

vii. Dehydrated in 70 % ethanol for 2 changes of 20 min each

viii. Dehydrated in 90 % ethanol for 2 changes of 10 min each

ix. Dehydrated in 100 % ethanol for 2 changes of 20 min each

x. Infiltrated with propylene oxide (1,2 epoxy propane) for 2 changes of 10 min each

xi. Infiltrated with propylene oxide/epoxy resin* mixture (50/50 v/v) for 1 hour

xii. Infiltrated with epoxy resin* overnight with caps removed from vials, allowing any remaining propylene oxide to evaporate

xiii. Tissue embedded in labelled capsules with freshly prepared resin*

xiv. Resin polymerised at 60°C for 48 hours

*Agar 100 resin, Agar Scientific

Appendix 3 – Reagent, bacteria and equipment suppliers

Agar Scientific: Agar Scientific, Stanstead, Essex, UK.

Amersham: Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Buckinghamshire, UK.

Anachem: Anachem Ltd, Anachem House, Charles Street, Luton, Bedfordshire, UK.

Animalcare: Animalcare Ltd. Common Road Dunnington York, UK.

Antec: Antec International, Sudbury, Suffolk, UK.

Bayer: Bayer plc. Animal Health Business Group, Bury St Edmunds, Suffolk, UK.

BBI: British Biocell International, Cardiff, UK.

BDH Ltd.: BDH Ltd, Poole, Dorset, UK.

Becton-Dickinson: BD Diagnostic systems, BD UK Ltd., Oxford, Oxfordshire, UK.

bioMérieux: bioMérieux UK Limited, Grafton Way, Basingstoke, Hampshire, UK.

Biometra: Biometra, Göttingen, Germany.

BioRad: Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, UK.

BOCM: BOCM Pauls Ltd., Ipswich, Suffolk, UK.

Cambio: Cambio, Cambridge, Cambridgeshire, UK.

CHROMagar: CHROMagar, Paris, France.

CPHL: Central Public Health Laboratory, 61 Colindale Avenue, London, UK.

Dako: Dako Ltd, Ely, Cambridgeshire, UK.

DNASTAR: DNASTAR Inc., Madison, Wisconsin, USA.

Dynal: Dynal A.S., Oslo, Norway.

Dynex: DYNEX Technologies, 14340 Sullyfield Circle, Chantilly, Virginia, USA.

Duran: Schott Glas Duran, Hattenbergstrasse 10, 55122 Mainz, Germany.

Eppendorf: Eppendorf UK Ltd., Cambridge, Cambridgeshire, UK.

Falcon: BD Biosciences Discovery Labware, BD UK Ltd., Oxford, Oxfordshire, UK.

Fort Dodge: Fort Dodge Animal Health, Southampton, Hampshire, UK.

Gibco: Gibco Invitrogen Corporation, Paisley, Renfrewshire, UK.

Heraeus: Heraeus Instruments, Inc., 111A Corporate Boulevade, South Plainfield, New Jersey, USA.

Invitrogen: 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF, UK.

ICT: Innovative Cell Technologies Inc., San Diego, California, USA.

Janssen: Janssen Animal Health, High Wycombe, Buckinghamshire, UK.

Lakeland Ltd.: Lakeland Limited, Windermere, Cumbria, UK.

Leitz: Leitz GmbH & Co. KG, Leitzstraße 2 73443, Oberkochen, Germany.

Life Technologies: Invitrogen Ltd, Paisley, Renfrewshire, UK.

London Resin Company: London Resin Company, Reading, Berkshire, UK.

Merial: Merial Animal Health Ltd., Harlow, Essex, UK.

MSE: Sanyo Gallenkamp PLC Monarch Way, Loughborough, UK.

National Diagnostics: National Diagnostics, Atlanta, Georgia, USA.

NCBI: National Center for Biotechnology Information, National Library of Medicine, Bethesda, Maryland, USA. Database access via URL: http://www.ncbi.nlm.nih.gov/

NCTC: National Collection of Type Cultures, CPHL (above), UK.

Nunc: Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY, USA.

Oxoid: Oxoid, Basingstoke, UK.

Pierce: Pierce Chemical Company, Rockford, Illinois, USA.

Promega: Promega UK, Southampton, Hampshire, UK.

R. A. Lamb: Raymond A. Lamb, Eastbourne, Sussex, UK.

Reichert: Leica Microsystems (UK) Ltd, Davy Avenue, Knowlhill, Milton Keynes, Buckinghamshire, UK.

Roche: Roche Products Limited, Vitamins and Fine Chemicals Division, Heanor, Derbyshire, UK.

Shandon: Shandon Scientific Ltd., Astmoor, Runcorn, Cheshire, UK.

Sigma: Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

SSL: SSL International Plc., Canute Court, Toft Road, Knutsford, Cheshire, UK.

Syngene: Syngene (Europe), Beacon House, Nuffield Road, Cambridge, UK.

Vivascience: Vivascience AG, Hannover, Germany.

WPA: Unit 22 - Cambridge Science Park, Milton Road, Cambridge, UK.

5'→3': 5 Prime → 3 Prime Inc., Boulder, Colorado, USA.

Appendix 4 – Summary of reports on the epidemiology of *E. coli* O157:H7 in cattle

Appendix table A: Reports on the epidemiology of *E. coli* O157:H7 in cattle. *Continued on pages 242 - 250. Notes page 250*

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Wells et al. 1991, USA	Survey, prevalence and follow-up. 22 herds.	1266 cattle	Faeces swabs, TSB pre-enrichment + SMAC plates	Dairy; adults, heifers and calves	Adults: 0.15%; Calves: 2.8% (45% of herds)	46 days (tested on days 0 and 46 only)	Plasmid profiling and toxin typing showed a different strain on one farm at second visit, 3 weeks later.
Chapman et al. 1993, UK	Survey, prevalence, abattoir	2103 cattle	Rectal swabs, CR-SMAC direct culture		4%		
Hancock et al. 1994, USA	Survey, prevalence. 60 herds.	3570 cattle	Faeces, TSB_V enrichment and subculture on SMAC	Beef herds (adults); Feedlots; Dairy herds (calves and adults)	Beef: 0.71% (16% herds); Feedlots 0.28% (8.3% farms); Dairy: 0.33% (10% herds)		Weaned calves had the highest prevalence.
Cray and Moon 1995, USA	Experimental, 10^{10}, 10^7, or 10^4 cfu oral inoculation	39 cattle	Faeces, daily to weekly up to 20 weeks p.i. Enrichment in TSB+ bile salts, subculture on SMAC.	17 calves (3-14 weeks); 22 adults (1-3 years).	Initially: 100% of 10^{10}; 40% of 10^7; 0% of 10^4 cfu-inoculated animals.	27 weeks	Detection of excretion related to dose. Detection in rumen and large intestine.
Garber et al. 1995, USA	Survey, prevalence, 31 herds.	965 cattle	Faeces, enrichment in TSB+ bile salts/novobiocin /acriflavin, subculture on MUG-SMAC.	Dairy; calves	<8 weeks: 1.4%; >8weeks: 4.8%		Risk factors identified for excretion: weaning, preweaning group housing.
Sanderson et al. 1995, USA	Experimental, 5 x 10^8 cfu oral inoculation, plus prevalence survey	355 cattle	Faecal swabs and faeces, 3x per week. TSB_{VC} enrichment, CT-SMAC subculture. Also IMS.	Calves, 1 week to 8 months.	2.3% to 5.4%, depending on detection technique.	43 days	Prevalence if results of 3 detection techniques are combined rises to 6.8%. IMS enrichment of 'neat' primary preparation only.

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Faith et al. 1996, USA	Survey, prevalence and follow-up over 6 months	560 cattle (prevalence); 517 (follow-up)	Rectal contents, mEC_N enrichment, subculture on (CT-)SMAC	Dairy, adults and calves	1.8% calves; 3-4% of all ages in O157-positive herds.		1% of water samples positive. PFGE shows certain O157 clones are common but not exclusive on a farm.
Besser et al. 1997 USA	Survey, longitudinal (1 year), 10 farms	1091 cattle, 4031 samples.	Monthly, rectal swabs, TSB_{VC} enrichment plus C-SMAC subculture; IMS additionally for some.	Dairy, adults and calves below 8 months.	4.9% animals over the year; 40% herds	4 months	Some samples O157-positive with IMS were negative with enrichment culture. PFGE shows strains vary between herds, animals and within animals concurrently and over time. Some PFGE types widespread within a herd.
Borie et al. 1997, Chile, (abstract only)	Survey, abattoir.	136 cattle, (120 pigs)	Faeces, unknown culture technique. DNA probes for Shiga toxin and *eaeA genes*.	Steers	O157 present, further characterisation unreported.		Abstract only seen.
Brown et al. 1997, USA	Experimental, 10^{10} cfu 5-strain oral inoculation, nal-resistant.	12 cattle	Daily, rectal contents, direct plus enrichment in TSB + bile salts & nal.	8 weeks old	Initially, 100%	27 days (euthanased)	Consistent recovery from rumen, ileum and colon.
Chapman et al. 1997, UK	Survey, prevalence, abattoir	4800 cattle (plus 1000 sheep)	Rectal contents, IMS subcultured onto CT-SMAC.		15.7% cattle. (2.2% sheep)		Temporal variation in prevalence: 36.8% in May to 4.8% in February.
Dargatz et al. 1997; Hancock et al. 1997c, USA	Survey, prevalence	11 881 samples (100 feedlots)	Faecal pats, shipped in transport medium or TSB_{VC}, enriched in TSB_{VC} or mEC_N, subcultured on CT-SMAC.	Feedlots.	1.58% pats; 25% pens; 63% premises. (0.2% pats positive for O157:H-)		Range of prevalence within lots is 0%-10%. No evidence of geographical variation. Highest prevalence in youngest stock, which had most recently experienced movement and dietary changes.
Hancock et al. 1997, USA	Survey, longitudinal, 14 herds (13 months)	10 832 samples	Monthly, rectal or faecal pat faeces samples. Culture tech not defined.	Dairy, adults and calves	0% - 2.6%; 1% of samples;		Temporal clustering of positive samples; positive herds tend to remain positive; seasonality observed.

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Hancock et al. 1997, USA	Survey, longitudinal	12 664 samples (36 herds)	Monthly, faecal pats, TSB_{VC} enrichment, subculture on CT-SMAC.	Dairy, postweaned heifers.	0%-26% pats in a herd. 0%-5.5% pats overall from any one herd; 75% herds overall.		Evidence of temporal clustering of detectable excretion (on 68% of visits no detection). Herd prevalence tends to be high or low, and stable over time.
Mechie et al. 1997, UK	Survey, longitudinal, 15 months, 1 farm	3593 samples, 106-166 cattle on each occasion	1-4 weekly, rectal swabs, IMS using BPW_{VCC} enrichment.	Dairy; calves and adults.	0-68%, varying over time, overall, 44% adults excreted	4 weeks	Peak prevalence in summer. Intermittent shedding with 2–51 week gaps. 74% of shedders were positive only once. Calves apparently excreted longest.
Rahn et al. 1997, Canada	Survey, prevalence, 8 farms O157-positive 1 year previously.	406 cattle	Rectal swab, MacConkey broth culture, subcultured on SMAC.	Dairy; adults and calves	0.5%		
Rice et al. 1997, USA	Survey, on-farm and 1 week later at abattoir	205 cattle (89 sampled both on-farm and at abattoir)	Rectal swabs, enriched in TSB_{VC}, subcultured on SMAC.	Dairy, cull cows	3.4% on-farm; 3.9% at abattoir.	1 week (equals duration of study)	O157 detected from 10.1% of 89 twice-sampled cattle on either or both sampling occasions.
Cray et al. 1998, USA	Experimental, 10^{10} and 10^{7} cfu oral inoculation.	13 cattle	Daily, faeces, TSB + bile salts & tellurite enrichment, SMAC subculture	3-4 months old			Excretion increased by fasting, but only at the time of inoculation, not after inoculation. Supports a transient role for the rumen in persistence.
Hancock et al. 1998b, USA	Survey, prevalence and longitudinal (12 farms)	2143 cattle	Bimonthly, faecal pats, TSB_{VC} enrichment, subculture on CT-SMAC.	Feedlot cattle and dairy heifers.	1.1%-6.1% prevalence. 100% of 12 farms positive.		O157 detected in 10/320 water troughs, positives from biofilm and water samples from any one trough do not correlate. PFGE shows same strains in cattle, water, environment and, on one farm, horse. True 'reservoir' status of cattle questioned.
Heuvelink et al. 1998a, Holland	Survey, prevalence, abattoir	353 cattle	Enrichment in mEC_N then either dilution and subculture on CT-SMAC or IMS	Adults and veal calves	11% (4% sheep)		IMS significantly more sensitive than direct subculture post-enrichment. Little overlap between pulsed field gel electrophoresis patterns of bovine isolates and 33 human isolates

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Heuvelink et al. 1998b, Holland	Survey, prevalence (10 farms), longitudinal (4 farms).	1152 cattle	Rectal swabs, 3-month intervals, IMS.	Dairy, all ages	Within-herd prevalence 0-60%.	3 months	Negative- to positive-excreting herd conversion observed. Cattle infrequently excreting on >1 sampling occasion. Summertime peak prevalence. Calves have highest prevalence.
Richards et al. 1998, UK	Survey, prevalence, using diagnostic faeces sample submissions.	6495 samples	Faeces, IMS with enrichment in BPW_{VCC}.	<6 months - adult	0.59% confirmed O157:H7 Shiga toxin positive		Calves significantly higher prevalence than older stock. Results subject to bias from the presence of gastrointestinal disease in many animals. Several O157:H- Shiga toxin+ strains isolated.
Shere et al. 1998, USA	Survey, longitudinal, 14 months, 4 farms.	402 cattle, 2737 bovine samples	Weekly, rectal contents, mEC_N enrichment + CT-SMAC plating.	Dairy; adults, plus 60 calves born during study period.	0% - 11%, varying between farms and sampling times.	16 weeks	Group housing of calves increased excretion risk. Water contamination correlated with excretion. Endemic strains persisted on farms, despite periodic detection of others. Seasonality not seen.
Cerqueira et al. 1999, Brazil	Survey, farms and abattoir.	197 cattle	Faeces, CT-SMAC culture.	Diary, beef.	1.5%		2/3 O157:H7 strains were negative on Verocytotoxicity assay.
Cizek et al. 1999, Czech Republic	Survey, longitudinal.	365 cattle	Rectal faeces, every 1 to 3 months, enrichment, IMS.	Calf fattening unit.	1% animals		19% of animals were excreting a non-sorbitol-fermenting, non-H7 *E. coli* O157.
Garber et al. 1999, USA	Survey, prevalence	4361 cattle	Rectal contents, mEC enrichment, dilution and CT-SMAC subculture.	Dairy, adults	1.2% (24% of herds)		Correlation of excretion with use of water troughs rather than individual drinkers.
Harmon et al. 1999, USA	Experimental, 10^{10} cfu, rumen cannula, nal resistant 5-strain inoculum	9 cattle	Faeces, daily or every other day TSB + bile salts & nal enrichment, SMAC subculture.	2-3 months old		25 days (duration of sampling)	Trial of effects of starvation on excretion. No effects observed.

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Hovde et al. 1999, USA	Experimental, 10^{10} cfu oral inoculation	8 cattle	Every 3-4 days, rectal contents, enrichment in TSB_{VCT}, subculture on CTM-SMAC.	1-2 year old steers		Average quoted, 39-42 days on forage.	Comparison between forage- and grain-feeding. Forage-fed animals excreted for much longer.
Rice et al. 1999, USA	Analysis of field O157:H7 strains by PFGE	(376 bacterial strains from 41 farms)	Up to 11 Xba I subtypes found per herd (up to 7 on any one occasion). Persistence of subtypes for 6-24 months on 4 farms. Closed dairy herds had similar number of subtypes as open herds; feedlots had more subtypes than dairy herds. Wide geographical range (up to 640km) of 5 subtypes.				
Sanderson et al. 1999, USA	Experimental, repeated inoculation at 1, 2 and 8 weeks old with 5 x 10^8 cfu nal-resistant strain	4 cattle	3x per week, faeces, direct culture on MacConkey agar + nalidixic acid.	Calves		43 days	Re-inoculation at increasing age resulted in progressively shorter excretion times, down to 3 days.
Akiba et al. 2000, Japan	Experimental, 10^9 cfu stomach tube inoculation	2 cattle	Daily, rectal contents, mEC_N enrichment then IMS.	8 weeks old		50 days	Loss of pO157 and *stx2*, and pulsed field gel electrophoresis changes observed over 63 days p.i.
Buchko et al. 2000a, Canada	Experimental, 10^9 cfu oral inoculation.	18 cattle	Faeces, 3x per week for 4 weeks, then weekly for 6 weeks. Direct culture and IMS.	Yearling steers.		67 days	O157 detected in 30 / 180 mouth swabs taken throughout the experiment.
Cobbold and Desmarchelier 2000, Australia	Survey, longitudinal (cohort study), 3 farms, 12 months	37 cattle, 588 samples.	1 pre-weaned, 3x during weaning, 3x post-weaning. Rectal swab, enriched in mEC+novobiocin, subcultured on mHC.	Dairy, calves and adults	Pre-wean: 1.3%; weanlings: 5.5%, heifers: 2.8%; adults 0.3%; overall: 1.9%	Not stated.	Weaned calves have the highest prevalence

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Magnuson et al. 2000, USA	Experimental, 10^{10} cfu oral inoculation.	8 cattle	Rectal contents, daily to weekly up to 30 d p.i. Enrichment in TSB_{VCT}, subculture on CTM-SMAC.	Heifers		69 days	No difference in excretion seen between forage- and grain-fed animals.
McDonough et al. 2000, USA	Survey, prevalence	1668 cattle	Faeces, 3 enrichment broths, subculture on CT-SMAC.	Dairy, cull cattle	1.3%		
Notario et al. 2000, Argentina	Survey	68 cattle	Rectal swab		O157 detected		Abstract only seen.
Price et al. 2000, USA	Experimental, 10^{10} cfu oral inoculation, antibiotic resistant strains.	3 cattle	Daily, faeces, direct culture or enrichment in BHI_{NRT} then subculture on Rainbow Agar O157.	8 weeks old		39 days	Comparative trial of wild-type and RpoS mutant strains
Sargeant et al. 2000, USA	Survey, longitudinal, 1 year	2058 cattle	Monthly, faeces, EEB, then IMS +/- post-IMS EEB enrichment and subculture on CT-SMAC.	Cow-calf herds, cows and calves	1.9% of animals over study period	No animal positive on >1 occasion	O157 detected in 1.5% of water samples.
Schurman et al. 2000, Canada	Survey, prevalence, abattoir.	1000 cattle	Rectal swab, Vero assay and subsequent culture	Beef slaughter-weight cattle	0.4%		O157 was the most common STEC identified.
Shinagawa et al. 2000, Japan	Survey, prevalence, farm and abattoir.	510 cattle	Faeces, mEC_N enrichment and IMS	Calves and adults	1%		

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Tkalcic et al. 2000, USA	Experimental, 10^{10} cfu oral inoculation, 5-strain mixture.	9 cattle	Daily faeces samples. Direct and enriched culture*	2-3 month old calves		35 days (duration of the experiment)	No difference in excretion between forage- and concentrate-fed animals.
Wray et al. 2000, UK	Experimental, 2×10^8 cfu oral inoculation	12 cattle	Daily/every other day, faeces, IMS using BPW_{VCC}.	Adults (6), calves < 1 month old (6)		44 days (adult)	Intermittent excretion to 58 days p.i. by calves. Adult excreting f or 44 days was inoculated at zero and 14 days p.i.
Besser et al. 2001, USA	Experiment, approx. 200 to 10^4 cfu oral inoculation, 9 nal^r strains.	17 cattle	Faeces, 3, 6, 10 days p.i., variable intervals thereafter. Enrichment in TSB_{nal}, TSB_{VC}; culture on TSA_{nal} or CT-SMAC. IMS as comparison.	12 to 29 week old calves.		70 consecutive days. Intermittently to euthanasia at 118 days p.i.	A dose as low as 210 cfu would induce persistent excretion of some strains in some calves. Indirect transmission between calves led to persistent excretion. IMS signficantly superior to other detection methods when <30 cfu/g present in faeces.
Bonardi et al. 2001, Italy	Survey, abattoir.	100 cattle	Rectal faeces, IMS	Feedlot and dairy, adults.	17% faeces samples over 10 months.		Prevalence varied widely month to month. Auto- and cross- contamination of carcasses demonstrated.
Chapman et al. 2001, England	Survey, single abattoir over 1 year.	4800 cattle (7200 sheep)	Rectal swabs, enrichment in BPW_{VCC} then IMS and subculture on CT-SMAC	Slaughter-weight animals	12.9% (7.4% of sheep)		Virtually all isolates possessed Shiga toxin, pO157 and *eaeA*. 92% of local human toxin/plasmid/phage type combinations were represented.
Conedera et al. 2001, Italy	Survey, prevalence and longitudinal (15 months)	341 cattle (prevalence); 92 (longitudinal)	Monthly and bi-monthly rectal swab, freeze-thawed, enriched BPW_V then IMS.	Dairy heifer-raising farm. 2 week-5month old calves, and 2.5 to 15 month old heifers.	3.8% (<1 month); 4.4% (<5 month); 2.7%-23.7% prevalence in older animals.	8 months	Animals not grazed. Peak prevalence in summer; monthly prevalence varied widely. 64% of animals positive on ≥1occasion(s). PFGE shows clonal stability over months in some animals. O157 detected in water troughs. Possible role of a few 'persistent excreters' discussed.

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Galland et al. 2001, USA	Survey, longitudinal, 11 months.	24 184 samples	Every 3 weeks, pools of 5 faecal pats. Broth enrichment and subculture onto SMAC.	Feedlot	0.19% of samples. (0.08% are typical O157:H7)		>50% of O157 isolates are LEE-negative. The importance of testing multiple colonies per plate, to increase sensitivity of detection, is noted.
Johnsen et al. 2001, Norway	Survey, abattoir and farm	Abattoir: 1541 cattle; Farm: 33 cattle	5g large intestinal content, BPW_{VCC} enrichment, then IMS.	Adults	0.19% abattoir; 0% on-farm.		
Jonsson et al. 2001, Sweden	Survey, longitudinal, 6 months	12 cattle; all O157- positive at start of study.	Monthly, faeces, IMS.	Calves, summer: 6 at pasture, 6 kept indoors	17% to 100% of indoor animals on any occasion, 0% of outdoor animals.	4 months.	Suggests substantial role for environment in animal excretion.
Kobayashi et al. 2001, Japan	Survey, prevalence	358 cattle	Rectal contents, direct culture on SMAC	Dairy, Adults and calves	0.28% (1 isolate)		
Lahti et al. 2001, Finland	Survey, abattoir.	1448 cattle	1g faeces, TSB pre-enrichment and IMS.	Adults	1.3%		1/19 isolates not H7, 1/19 isolates toxin-negative.
Nielsen 2001, Denmark	Survey, herd prevalence, plus longitudinal (6 months) of 8 positive herds.	Herd prevalence: up to 3000 cattle. Longitudinal: up to 1440 cattle	3-monthly, faeces, IMS with BPW pre-enrichment, subcultured onto CT-SMAC.	Dairy, calves and cows	10/60 (17%) herds positive. Means in positive herds: 6.9% -16.3%		5 or 6 of the initially positive herds were positive at each of the three follow-up visits. The resident strain was stable (by PFGE) for 9-21 months on several farms. New strains were seen on at least two farms.
Synge et al. 2001, Scotland	Surveys, prevalence and longitudinal (3 years)	**Prev.:** 14 849 samples (952 groups) **Long.:** 9256 samples (32 farms)	Monthly, faecal pats, pre-enrichment in BPW, then IMS and subculture on CT-SMAC.	**Prev.:** Beef, 12-30 months. **Long.:** Beef suckler cows.	**Prev.:** 7.9% animals, 22.8 groups (calculated) **Long.:** 4.2% samples.	8 months per herd (longitudinal study)	Phage type 21/28 heavily predominant. Excretion in suckler herds associated with: housing, group number change, presence of dogs and wild geese. Excretion in beef finishers associated with housing.

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Van Donkersgoed et al. 2001, Canada	Survey, seasonal prevalence (1 year) and longitudinal (6 months).	c. 10 000 faeces samples	Monthly, faecal pat swabs, TSB_{VC} enrichment and subculture on CT-SMAC.	Feedlot, arrival to slaughter	0.8% of pens positive over the year.		Longitudinal study showed no faecal O157, but O157 detected in 8%-22% water troughs and 2% of feed bunks. Many PFGE types seen, but a few appear 'endemic'
Vold et al. 2001, Norway	Survey, prevalence.	504 cattle	1g faeces, freeze-thawed, Gram negative broth enrichment and IMS.	Not defined (all imported)	4.6% -		Many of the O157:H7 isolates were toxin-negative.
Cornick et al. 2002, USA	Experiment, 2×10^{10} cfu dual oral inoculation with O157:H7 and isogenic *eaeA* mutant.	8 cattle	3, 7, 30, 60 days p.i.; faeces, direct plus enrichment (TSB+bile salts) culture onto MacConkey's. IMS also.	Yearlings		Unclear, both parent and mutant strains detected up to 2 month p.i. in some animals.	The level of excretion of the intimin mutant was significantly lower than that of the parent strain at all sampling points, when co-inoculated. Sheep data from the same report correlates with the cattle data.
Grauke et al. 2002, USA	Experiment, 3×10^{8} or 4×10^{10} cfu via gastric tube or rumen cannula. Human-derived O157:H7 strains.	22 cattle	Faeces, (plus rumen and duodenal contents of 4 animals), enriched in TSB_{VCT}, subcultured on CTVM-SMAC.	1 year old steers and heifers, 3-5 year old steers.	100% immediately post-inoculation	69 days	No difference between 10^{8} and 10^{10} cfu doses in excretion levels. Animals naturally clustered into: <1week excretion, about 1 month excretion, and >2 month excretion. This was independent of strain, dose, age, breed or prior exposure. No evidence of persistence in rumen or duodenum.
Paiba et al. 2002, UK	Survey, prevalence, abattoirs (118). Samples taken over 12 months.	4173 cattle (4189 sheep)	Rectal contents, IMS using BPW pre-enrichment, subculture on CT-SMAC.	18-30 months	4.7% (1.7% sheep)		Summer peak prevalence for cattle and sheep. Predominant phage types (cattle 2, 21/28, 8; sheep 4, 32) reflect those commonly associated with UK human infection.

Appendix table A continued

Reference, country	Survey or experiment (plus details)	Number of cattle or faecal pats sampled	Samples: frequency, type, detection method(s)	Management systems; Animal age ranges	Prevalence (%) or proportion of excreting animals	Longest individual excretion	Comments
Naylor et al., UK / USA	Experiment, 10^9 cfu oral inoculation, nal^r bovine strains, euthanasia 14-28 days p.i. Also 1 persistently-excreting field case.	15 calves	Faeces, and intestinal contents *post mortem*. Unenriched on $SMAC_{nal}$ or enrichment in LB_{nal} (experimental) or LB_{CT}, then culture on SMAC.	8- 14 weeks at inoculation, naturally-colonised steer 12 months old.		All experimental animals excreting for 2-4 weeks when euthanased.	Demonstrated marked increase in concentration of *E. coli* O157:H7 in terminal rectum towards anus, and adherence of O157 organisms at recto-anal junction. Also, coating of faeces with the organism.

Notes for Appendix table A:

BHI_{NRT}: Brain-heart infusion broth plus novobiocin, rifampicin and tellurite.

BPW: Buffered peptone water.
BPW_V: BPW plus vancomycin
BPW_{VCC}: BPW plus vancomycin, cefixime and cefsulodin

EEB: EHEC enrichment broth

IMS: Immunomagnetic separation

LB_{nal}: Luria-Bertani broth plus nalidixic acid
LB_{CT}: Luria-Bertani broth plus cefixime and tellurite

mEC: modified *E. coli* broth
mEC_N: modified *E. coli* broth plus novobiocin
mHC: modified Haemorrhagic colitis medium

nal: nalidixic acid

PFGE: pulsed-field gel electrophoresis

p.i.: post-inoculation

SMAC: sorbitol-MacConkey agar
$SMAC_{nal}$: SMAC plus nalidixic acid
C-SMAC: SMAC plus cefixime
CT-SMAC: SMAC plus cefixime and tellurite
CR-SMAC: SMAC plus cefixime and rhamnose
CTM-SMAC: SMAC plus cefixime, tellurite and 4-methylumbelliferyl-β-**D**-glucuronide (MUG)
CTVM-SMAC: SMAC plus cefixime, tellurite, vancomycin and MUG

TSA_{nal}: Tryptone soya agar plus nalidixic acid
TSB: Tryptone soya broth
TSB_{nal}: Tryptone soya broth plus nalidixic acid
TSB_{VC}: TSB plus vancomycin and cefixime
TSB_{VCT}: TSB plus vancomycin, cefixime and tellurite

Appendix 5 – Large intestine surface area calculation

Appendix table B: Approximate macroscopic surface area of large intestine

Region	Length* (cm)	Circumference* (cm)	Area (cm^2)
Caecum	22	8	176
Proximal ascending. colon	30	8	240
Spiral colon	260	2.5	650
Descending colon	45	2	90
Rectum	15	3	45
Total large intestine			1201

* Measurements were taken from the intestine of a clinically normal freshly-killed six-month old sheep at the end of a liver fluke study

Appendix 6 – Publications arising from the present studies

Wales, A. D., F. A. Clifton-Hadley, A. L. Cookson, M. P. Dibb-Fuller, R. M. LaRagione, K. A. Sprigings, G. R. Pearson and M. J. Woodward (2001). Experimental infection of six-month old lambs with *Escherichia coli* O157:H7. *Vet Rec* **148** (20): 630-631.

Wales, A. D., G. R. Pearson, A. M. Skuse, J. M. Roe, C. M. Hayes, A. L. Cookson and M. J. Woodward (2001). Attaching and effacing lesions caused by *Escherichia coli* O157:H7 in experimentally inoculated neonatal lambs. *J Med Microbiol* **50** (9): 752-758.

Wales, A. D., F. A. Clifton-Hadley, A. L. Cookson, M. P. Dibb-Fuller, R. M. LaRagione, K. A. Sprigings, G. R. Pearson and M. J. Woodward (2002). Production of attaching-effacing lesions in ligated large intestine loops of 6-month-old sheep by *Escherichia coli* O157:H7. *J Med Microbiol* **51** (9): 755-763.

Cookson, A.L., A.D. Wales, J.M. Roe, C.M. Hayes, G.R. Pearson and M.J. Woodward (2002). Variation in the persistence of *Escherichia coli* O157:H7 in experimentally inoculated six-week-old conventional lambs. *J Med Microbiol* **51** (12): 1032-1040.

Appendix 7 – Contents of cited World Wide Web pages

1) **Anon,** accessed 2002. VT-Producing *Escherichia coli* O157 Strains Examined by PHLS LEP. England and Wales 1992 - 2001 quarterly. Public Health Laboratory Service. http://www.phls.org.uk/facts/Gastro/ecoli/ecoliQua.htm

VT-PRODUCING *Escherichia coli* O157 Strains Examined by PHLS LEP
England and Wales
1992 - 2001 quarterly

	1st Quarter	2nd Quarter	3rd Quarter	4th Quarter	TOTAL
1992	38	149	205	78	**470**
1993	38	94	185	68	**385**
1994	33	94	220	64	**411**
1995	41	143	413	195	**792**
1996	44	132	300	184	**660**
1997	89	215	558	225	**1087**
1998	140	181	364	205	**890**
1999	186	239	484	175	**1084**
2000	65	248	420	163	**896**
2001*	73	161	352	182	**768**

* Provisional data
Source: PHLS Laboratory of Enteric Pathogens

2) **Skuse, A.M.,** Accessed 2002a. Retrieval of tissue from histological wax blocks for E.M. University of Bristol Comparative Pathology Laboratory. http://www.bris.ac.uk/Depts/PathAndMicro/CPL/emtechs.htm#Retrieval Of Tissues From Wax

RETRIEVAL OF TISSUE FROM HISTOLOGICAL WAX BLOCKS FOR E.M.
In some cases an area of interest, which may not be discovered by simply processing more tissue, can be retrieved from the wax block.

1. Identify the area of interest on the microscope slide by ringing it with a marker pen.
2. Match the area marked on the slide against the specimen in the wax block and cut around it with a razor blade.
3. Cut a few millimetres into the surface of the wax block all around the marked area.
4. Carefully lever out the piece of tissue.
5. Cut the piece of tissue into suitable sized blocks making sure that orientation can be recognised later by cutting so that one dimension is greater than the other two.
6. Place the tissue into a glass processing vial and fill it with a suitable wax solvent (Histo-Clear® or xylene) and leave for 24 hours, (preferably on a rotating mixer).
7. Place tissue into 100% ethanol for 2 changes of 1 hour each.
8. Place tissue into 90% ethanol for 2 changes of 30 minutes each.
9. Place tissue into 70% ethanol for 2 changes of 30 minutes each.
10. Place tissue into 0.1M sodium cacodylate buffer for 2 changes of 30 minutes each.
11. Continue with usual processing schedule for E.M. specimens.
12. The flat (previously cut) surface will be embedded facing the end of the embedding capsule so that the required area is accessible in the finished block.

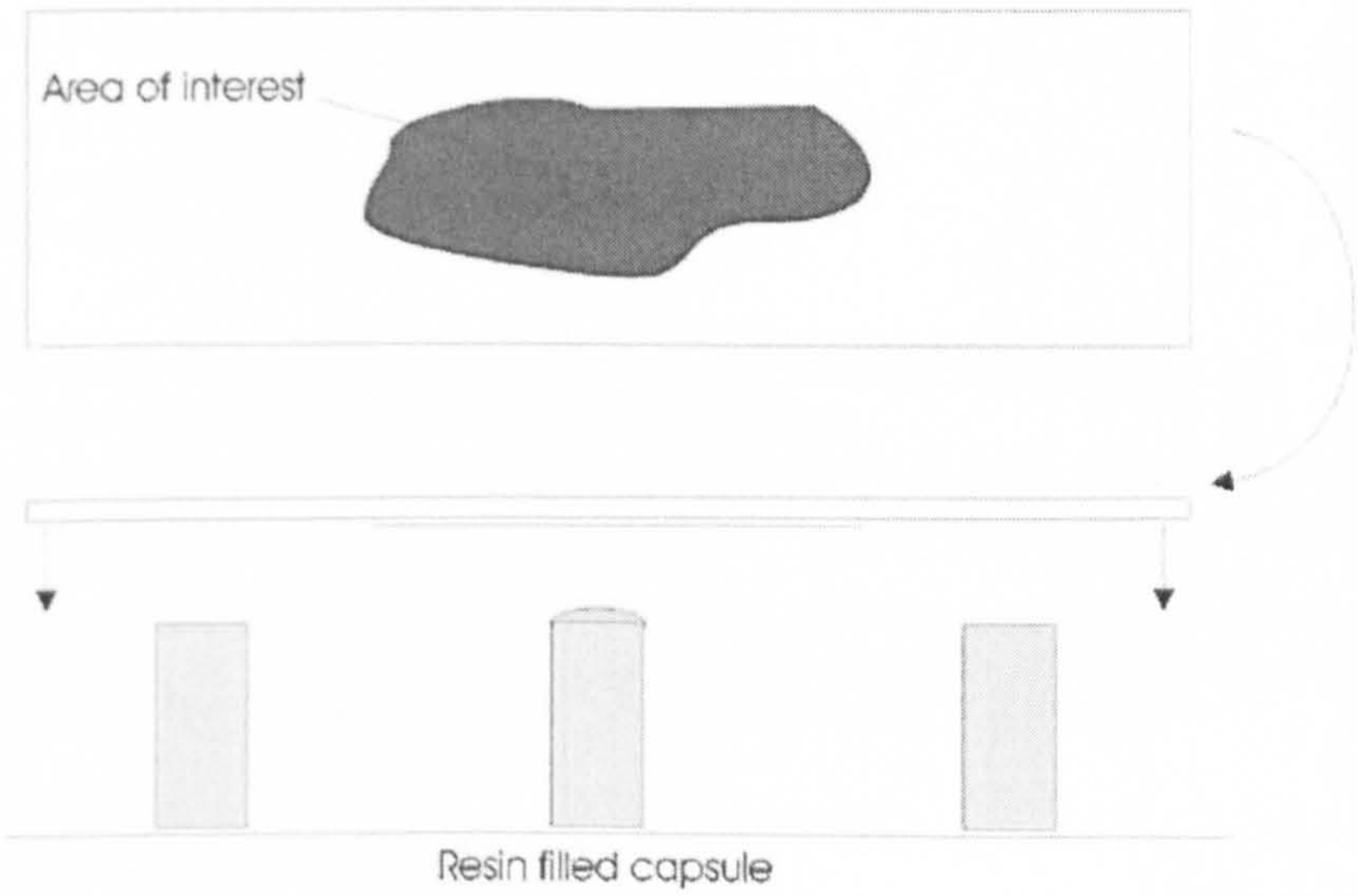
3) **Skuse, A.M.,** Accessed 2002b. Removal of tissue section material from glass slides for EM. University of Bristol Comparative Pathology Laboratory. http://www.bris.ac.uk/Depts/PathAndMicro/CPL/emtechs.htm#PopOff

REMOVAL OF TISSUE SECTION MATERIAL FROM GLASS SLIDES FOR EM

In some cases the area of interest in a histological section is so rare that finding a similar area by removal of tissue from the wax block will not give the required result. In this case it is possible to retrieve the actual tissue from the glass slide for EM.

1. Ring the area of interest on the top surface of the slide and then mirror that ring on the reverse of the slide with a diamond tipped pen. This will allow the correct positioning of the slide later.
2. Remove the coverslip by soaking the slide in Histoclear® or xylene until the DPX mountant is loosened.
3. Soak the slide in Histoclear® or xylene for a further few hours to remove all the mountant.
4. Place the slide in 100% alcohol for two or three changes of 30-60 minutes each.
5. Place in a sealed container of LR White® resin for two or three changes of at least 12 hours each.
6. **Either:**
   - Make up sufficient quantity of LR White® with accelerator to fill one or two EM embedding capsules. 10ml resin : 1 drop accelerator is a convenient quantity.
   - DO NOT coat the inside of the capsule with extra accelerator.
   - Fill the EM capsule completely to the brim with resin mixture so that it is convex at the surface. (See diagram.)
   - Place two empty capsules either side of it on a flat surface for support.
   - Remove the slide from the coplin jar and drain off as much excess resin as possible.
   - Place the slide section side down on top of the resin mixture making sure that the marked area of interest is in the centre of the resin.
   - Allow to polymerise for 15-30 minutes or until hardened.

     Or:
   - Use resin without accelerator. This may be better for any sample that requires immunocytochemistry at a later stage as the use of accelerator generates high temperatures in the resin.
   - Use a multi-capsule block as single capsules tend to distort during curing and the block gives more support.
   - Fill 1 capsule completely to the brim with resin so that it is convex at the surface. (See diagram.)
   - Remove the slide from the coplin jar and drain off as much excess resin as possible.
   - Place the slide section side down on top of the resin making sure that the marked area of interest is in the centre of the resin.
   - Polymerise at 50°C for 12-24 hours.



7. Spray the reverse of the slide liberally with freezer spray. After a few seconds a crack will often be heard which denotes the partial separation of the resin from the slide.
8. Snap the capsule from the slide, some force may be necessary. The section of tissue should be embedded in the resin.
9. Section as soon as possible. The resin has a tendancy to distort gradually after polymerisation which prevents a whole section being taken. Trimming should be kept to an absolute minimum as the tissue is right at the surface of the block and is very thin.

4) **Skuse, A.M.,** Accessed 2002c. Immunogold staining technique. University of Bristol Comparative Pathology Laboratory. http://www.bris.ac.uk/Depts/PathAndMicro/CPL/emtechs.htm#Immunogold

IMMUNOGOLD STAINING TECHNIQUE

1. Cut sections (preferably processed into acrylic resin) and mount on inert grids such as nickel or gold.
2. Rinse grids in distilled water for 10 minutes.
3. Incubate in pH 7.4 T.B.S. (tris buffered saline) containing 5% normal serum for 30 minutes. (Serum from same animal as secondary antibody). The concentration of the normal serum may have to be increased to up to 50% to prevent background signal.
4. Incubate in specific primary antibody diluted 1 in 5 with pH 7.4 T.B.S., including 0.1% bovine serum albumin, for 30 minutes. (Check pH after preparation).
5. Wash grids in two changes of pH 7.4 T.B.S. for 5 minutes each, then two changes of pH 8.2 T.B.S. for 5 minutes each.
6. Incubate with immunogold conjugated secondary antibody diluted 1 in 50 with pH 8.2 T.B.S., including 0.8% bovine serum albumin, for 1.5 hours.
7. Wash grids in pH 8.2 T.B.S. for 5 minutes x 2.
8. Post fix grids in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 15 minutes.
9. Wash grids in two changes of distilled water for 5 minutes each.
10. Stain grids with uranyl acetate and lead citrate. (If using LR White® resin stain in aqueous uranyl acetate.)

TRIS BUFFERS

Tris buffer 0.05M

- Dissolve 6.1g tris(hydroxymethyl)methylamine in 50mls of distilled water.
- Add 37mls of 1M HCl.
- Dilute to a total volume of 1 litre with distilled water.
- pH should be 7.4 at 25°C, adjust with 1M HCl if necessary.

Tris buffered saline (T.B.S.) pH 7.4

- 0.05M tris buffer pH 7.4 (as above) - 100mls
- NaCl - to 2.5% w/v
- Triton X-100 - to 0.2% v/v

Tris buffered saline (T.B.S.) pH 8.2

- As above but adjust pH of buffer to 8.2 with 1M NaOH.

CONTROLS

1. Omit the primary antibody by leaving grids in the wash/block solution at step 3 and continuing to step 5. (Checks the secondary and substrate).
2. Replace the primary antibody with another but inappropriate antibody. (Checks the primary).
3. Replace the primary antibody with normal (non-immune) serum obtained from the same animal as the primary. (Checks the primary).
4. For immuno-gold detection of bacterial antigens dried onto formvar grids it may be necessary to block at step 3. with more concentrated normal serum as the formvar may absorb proteins non-specifically. (Putting on concentrated normal serum blocks this capacity).

5) **Various,** Accessed 2002. TrEMBL database accession number 069412: ESPA. ExPASy. http://ca.expasy.org/cgi-bin/niceprot.pl?O69412

NiceProt View of TrEMBL: O69412

General information about the entry

Entry name	O69412
Primary accession number	O69412
Secondary accession numbers	None
Entered in TrEMBL in	Release 07, August 1998
Sequence was last modified in	Release 07, August 1998
Annotations were last modified in	Release 21, June 2002

Name and origin of the protein

Protein name	ESPA protein
Synonym	Secreted protein EspA
Gene name	ESPA or Z5107 or ECS4556

From Escherichia coli [TaxID: 562]
Escherichia coli O157:H7 [TaxID: 83334]

Taxonomy Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia.

References

[1]
SEQUENCE FROM NUCLEIC ACID.
SPECIES=E.coli; **STRAIN**=EHEC EDL933; Kresse A.U., Ebel F., Deibel C., Chakraborty T., Guzman C.A.; Submitted (MAY-1997) to the EMBL/GenBank/DDBJ databases.

[2]
SEQUENCE FROM NUCLEIC ACID.
SPECIES=E.coli; **STRAIN**=EDL933; MEDLINE=98339885; PubMed=9673266; Perna N.T., Mayhew G.F., Posfai G., Elliott S., Donnenberg M.S., Kaper J.B., Blattner F.R.; "Molecular evolution of a pathogenicity island from enterohemorrhagic Escherichia coli O157:H7."; Infect. Immun. 66:3810-3817(1998).

[3]
SEQUENCE FROM NUCLEIC ACID.
SPECIES=E.coli O157:H7; **STRAIN**=O157:H7 / EDL933 / ATCC 700927;
MEDLINE=21074935; PubMed=11206551; Perna N.T., Plunkett G. III, Burland V., Mau B., Glasner J.D., Rose D.J., Mayhew G.F., Evans P.S., Gregor J., Kirkpatrick H.A., Posfai G., Hackett J., Klink S., Boutin A., Shao Y., Miller L., Grotbeck E.J., Davis N.W., Lim A., Dimalanta E.T., Potamousis K., Apodaca J., Anantharaman T.S., Lin J., Yen G., Schwartz D.C., Welch R.A., Blattner F.R.; "Genome sequence of enterohaemorrhagic Escherichia coli O157:H7."; Nature 409:529-533(2001).

[4]
SEQUENCE FROM NUCLEIC ACID.
SPECIES=E.coli O157:H7; **STRAIN**=O157:H7 / RIMD 0509952; MEDLINE=21156231; PubMed=11258796; Hayashi T., Makino K., Ohnishi M., Kurokawa K., Ishii K., Yokoyama K., Han C.-G., Ohtsubo E., Nakayama K., Murata T., Tanaka M., Tobe T., Iida T., Takami H., Honda T., Sasakawa C., Ogasawara N., Yasunaga T., Kuhara S., Shiba T., Hattori M., Shinagawa H.; "Complete genome sequence of enterohemorrhagic Escherichia coli O157:H7 and genomic comparison with a laboratory strain K-12."; DNA Res. 8:11-22(2001).

Comments

None

Cross-references

EMBL	Y13068; CAA73506.1; -. AF071034; AAC31501.1; -. AE005594; AAG58820.1; -. AP002566; BAB37979.1; -.
InterPro	IPR005095; EspA.
Pfam	PF03433; ESPA; 1.
Implicit links to	CMR; ProDom; ProtoMap; PRESAGE; ModBase; SWISS-2DPAGE.

Keywords

Complete proteome.

Features

None

Sequence information

Length: **192 AA**
Molecular weight: **20574 Da**
CRC64: **D536395FAB782C96** [This is a checksum on the sequence]

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        10         20         30         40         50         60
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MDTSNATSVV NVSASSSTST IYDLGNMSKD EVVKLFEELG VFQAAILMFS YMYQAQSNLS

        70         80         90        100        110        120
        |          |          |          |          |          |
IAKFADMNEA SKASTTAQKM ANLVDAKIAD VQSSTDKNAK AKLPQDVIDY INDPRNDISV

       130        140        150        160        170        180
        |          |          |          |          |          |
TGIRDLSGDL SAGDLQTVKA AISAKANNLT TVVNNSQLEI QQMSNTLNLL TSARSDVQSL

       190
        |
QYRTISAISL GK
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